

Electrochemical fingerprints of sequential double-regenerative enzymatic mechanisms in protein-film voltammetry

Milkica Janeva · Pavlinka Kokoskarova · Rubin Gulaboski 

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Abstract

Electrochemical transformations of many lipophilic redox proteins and enzymes proceed through sequential two-step electron-transfer pathways that are frequently coupled with regenerative catalytic reactions accompanying both electron-transfer steps. Although protein-film voltammetry (PFV) provides a powerful experimental platform for direct investigation of such systems, theoretical descriptions of surface-confined double-regenerative mechanisms remain largely unexplored. In this work, we present, for the first time, a comprehensive theoretical framework describing a two-step surface-confined double-regenerative mechanism under square-wave protein-film voltammetry conditions. Systematic theoretical analysis demonstrates how the interplay between heterogeneous electron-transfer kinetics and successive regenerative catalytic reactions governs the shape, position, intensity, and kinetic characteristics of the corresponding square-wave voltammetric responses. Simulations reveal a broad spectrum of characteristic electrochemical fingerprints that can be directly associated with variations in the intrinsic kinetic parameters of both electron-transfer and catalytic steps. The developed framework provides a robust basis for mechanistic interpretation of experimentally recorded protein-film voltammograms and establishes practical diagnostic criteria for distinguishing different kinetic regimes. Furthermore, the proposed approach offers a reliable platform for developing experimental protocols aimed at quantitative extraction of electron-transfer and catalytic rate constants, thereby enabling evaluation of enzymatic activity and reaction pathways of various redox proteins and enzymes. The presented model considerably expands the theoretical foundation of protein-film square-wave voltammetry applied to surface-confined redox proteins and enzymes.

Keywords Protein-film voltammetry · EC' catalytic mechanism · Surface EC'EC'' mechanism · Square-wave voltammetry · Enzymatic voltammetry

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Introduction

Surface-confined electrode reactions are seen as fundamental processes in modern electrochemistry because they provide direct insight into electron-transfer processes occurring at various interfaces, which are ubiquitous in biological, chemical, and energy-related systems^{1–4}. In particular, surface redox reactions are essential for understanding the electrochemical behavior of lipophilic redox enzymes and proteins, whose active sites are embedded within hydrophobic domains and operate in close proximity to biological membranes⁵. Representative examples include lipophilic quinone-processing enzymes, many biochemical systems containing lipophilic polyphenol moieties, haem-containing proteins, iron–sulfur proteins, and some lipophilic components of the mitochondrial respiratory chain, where electron transfer occurs through sequential redox steps coupled to chemical transformations^{3,5–8}.

A major breakthrough in electrochemical probing of such systems was achieved with the development of protein-film voltammetry (PFV), introduced by Armstrong approximately three decades ago^{7,9,10}. This technique enables direct electrochemical probing of redox enzymes activity by immobilizing micromolar amounts of protein onto lipophilic, predominantly graphite-based electrodes⁹. By eliminating the need for mediators and avoiding the mass transport via diffusion, PFV made it possible to resolve intrinsic redox properties, electron-transfer kinetics, catalytic turnover, and thermodynamic parameters of many redox enzymes under well-defined electrochemical control^{7,9–13}. Since then, PFV has become a widely explored approach for studying membrane-associated proteins and many other lipophilic redox systems^{9–13}.

Catalytic mechanisms in such surface-confined lipophilic redox systems are of particular importance^{3,10,11,12,13,14,15,16,17,18}, as they provide access to key mechanistic and kinetic parameters governing biological function. Analysis of catalytic voltammetric responses allows extraction of formal redox potentials, electron-transfer rate constants, chemical regeneration rates, and catalytic efficiencies, all of which are directly relevant to enzyme activity under physiological conditions^{3,9–14}.

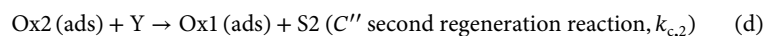
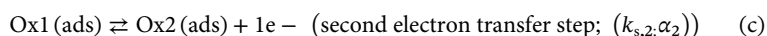
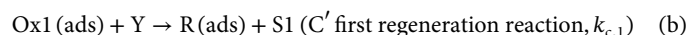
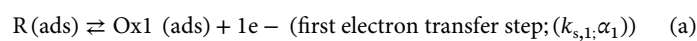
In many biologically relevant systems, the electrochemical response is not governed by a single electron-transfer step, but instead involves multiple consecutive redox transformations, each potentially coupled to various chemical reactions^{3,8,19–28}. It is already well recognized that a large number of lipophilic enzymes and redox proteins undergo two consecutive electron-transfer steps, such as those involving quinone/quinol couples^{21,24,25,26}, flavin cofactors^{3,23}, nicotinamide-related systems²³, and multi-center lipophilic metalloenzymes^{10–14}. Importantly, both electron-transfer steps may be coupled to regenerative chemical reactions, leading to complex double catalytic surface behavior (or surface EC'EC'' mechanism). Such mechanisms are especially relevant in biological electron-transfer chains^{14,21,22,23}, where chemical regeneration of redox molecules is an inherent part of proper cell functioning.

Under conditions of square-wave voltammetry (SWV)⁴, which is particularly sensitive to kinetic and mechanistic features due to its inherent discrimination between faradaic and capacitive currents, complex electrode mechanisms can be resolved with high sensitivity. Due to the widespread importance of surface-confined redox processes in bioelectrochemistry^{1,2,3,4}, a theoretical treatment of the two-step surface double regenerative mechanism is of utmost importance. In the present work, we address, for the first time, a comprehensive theoretical analysis and simulation of two-step surface EC'EC'' mechanism under square-wave protein-film voltammetry conditions. The voltammetric scenarios explored here reveal distinct features arising from the interplay between kinetics of sequential electron-transfer steps and the kinetics of regenerative chemical reactions involved. This mechanism is highly relevant for the interpretation of SWV responses of lipophilic redox enzymes, surface-bound cofactors, protein films, and biomimetic catalytic interfaces, where multi-electron transformations and catalytic regeneration occur at electrode surfaces^{5–7}. By establishing clear voltammetric fingerprints and parameter dependencies for this complex mechanism, the present study provides a new theoretical framework for analyzing and interpreting SWV data of surface-confined biological and bioinspired redox systems. In addition, the elaborated

model extends the applicability of square-wave protein-film voltammetry for mechanistic investigations of complex redox enzymatic systems in modern bioelectrochemistry.

Description of the mathematical model

The surface EC'EC'' mechanism describes a two-step consecutive electrochemical transformation of firmly adsorbed redox species, in which the product of each electron-transfer step subsequently participates in an irreversible chemical reaction that regenerates the species of preceding redox state. The electrochemical process is initiated by a one-electron oxidation of the initial redox-active species R(ads) (which is assumed to be the only electroactive reactant present in the electrochemical cell at the start of the experiment, uniformly adsorbed at the surface of working electrode), leading to the formation of a stable intermediate species Ox1(ads) via an one-electron-transfer step. Immediately after its formation, adsorbed redox species Ox1(ads) can undergo an irreversible chemical reaction with a reagent “Y” (assumed to be present in excess in the voltammetric cell), thereby regenerating the initial reactant R(ads) through a first catalytic pathway characterized by the rate constant $k_{c,1}$ (this is the EC' step in reaction scheme I). In parallel, Ox1(ads) species may undergo a second electrochemical oxidation via an additional one-electron transfer, resulting in the formation of the final stable and strongly adsorbed product Ox2(ads) (for simplicity, the charges of all species are omitted). The newly formed species Ox2(ads) can also participate in an irreversible chemical reaction with the same reagent “Y”, while regenerating the intermediate redox-active species Ox1(ads). This second regenerative pathway, governed by the rate constant $k_{c,2}$, constitutes the second, or the EC'' step of the overall mechanism (I). Collectively, these coupled electrochemical and chemical processes establish a double regenerative surface electrochemical cycle, in which continuous feedback occurs between the two electron-transfer steps, as summarized by reaction sequences (a)–(d) in the Reaction scheme (I).



Reaction scheme (I) Reaction scheme illustrating sequences (a–d) of the investigated surface EC'EC'' electrochemical mechanism, consisting of two successive electron-transfer steps each coupled to a corresponding regenerative chemical reaction. The kinetics of first and second electron-transfer steps are defined by the standard rate constants $k_{s,1}$, $k_{s,2}$ and the electron transfer coefficients α_1 , α_2 , while the regenerative steps are governed by the catalytic rate constants $k_{c,1}$ and $k_{c,2}$. Symbols S1 and S2 in reaction sequences (b) and (d) denote side products formed during the respective chemical regeneration steps; these species are assumed electrochemically inactive and do not participate in any electrode transformations within the applied potential window.

The mathematical algorithm that describes electrochemical mechanism presented by sequences (a)–(d) in reaction scheme (I) is based on a series of differential equations that account for the time-dependent surface concentrations of all redox active species involved in (I), and the contribution of chemical reactions as processes that govern the time-dependent surface concentrations of “R”, “Ox1” and “Ox2”. It is supposed in the mathematical model that all redox species are strongly adsorbed (“ads”) at the surface of working electrode, and no electrode transformation occurs via mass transfer by diffusion. In addition, no interaction between any of the adsorbed redox species is assumed to take place. For each of the redox species involved in the electrode transformation, the rate of change of corresponding concentration is described by a balance between time-dependent surface concentration and the

appropriate chemical term that accounts for the contribution of corresponding regenerative reactions. The surface concentration of the reduced species “R” increases due to the regenerative EC' reaction in which “Ox1” is converted back to “R”, and this contribution is included through the term “ $k_{c,1} \cdot \Gamma(\text{Ox1})$ ” (by Γ is assigned the surface concentration expressed in mol/cm²). The intermediate redox species “Ox1” is produced electrochemically at the electrode surface, consumed via the first regenerative chemical reaction (rate constant of first regenerative step $k_{c,1}$), and regenerated again in the second step through the reaction of the final species “Ox2”, governed by rate constant related to second irreversible chemical step ($k_{c,2}$). The final firmly adsorbed redox species “Ox2” is formed at the electrode surface in the second electron-transfer step, while it is depleted by the second chemical reaction with rate defined with the term “ $k_{c,2} \cdot \Gamma(\text{Ox2})$ ”. The differential Eq. (1) to (3) are relevant expressions that link the time-dependent surface concentrations of all redox species involved in (I) with the produces current, while taking into account the contribution of corresponding regenerative chemical reactions that together define the mathematical algorithm of the double-regenerative EC'EC'' surface electrochemical mechanism.

$$\frac{d\Gamma(\text{R})}{dt} = \frac{-I_1}{n \cdot F \cdot A} + k_{c,1} \cdot \Gamma(\text{Ox1}) \quad (1)$$

$$\frac{d\Gamma(\text{Ox1})}{dt} = \frac{I_1}{n \cdot F \cdot A} - \frac{I_2}{n \cdot F \cdot A} - k_{c,1} \cdot \Gamma(\text{Ox1}) + k_{c,2} \cdot \Gamma(\text{Ox2}) \quad (2)$$

$$\frac{d\Gamma(\text{Ox2})}{dt} = \frac{I_2}{n \cdot F \cdot A} - k_{c,2} \cdot \Gamma(\text{Ox2}) \quad (3)$$

It should be noted that the model can also accommodate two different regenerative substrates, Y and Z, for example, selectively coupled to the first and second electron-transfer steps, respectively. Such a situation may be particularly relevant for redox proteins and other highly selective systems. Providing that both substrates are present in excess and exhibit strict specificity toward the corresponding redox states, the mathematical treatment and the principal outcomes of the model remain unchanged, while the two pseudo-first-order rate constants can be varied independently through the concentrations of Y and Z.

Initial and boundary conditions

Differential Eq. (1) to (3) have been solved at following boundary conditions:

The reduced form “R” exists initially as only redox form adsorbed uniformly at working electrode surface, and its concentration in $t=0$ equals the total concentration of all surface active redox species defined as $\Gamma^*(\text{R})$, i.e.,

$$\Gamma(\text{R})_{(t=0)} = \Gamma^*(\text{R}) \quad (4)$$

The intermediate redox species “Ox1” is formed only electrochemically at electrode surface during the first electron-transfer step, so its surface concentration remains zero at $t=0$, i.e.:

$$\Gamma(\text{Ox1})_{(t=0)} = 0 \quad (5)$$

The final species of electrode transformation “Ox2” is also generated solely at the electrode surface, so it does not exist at $t=0$, i.e.:

$$\Gamma(\text{Ox2})_{(t=0)} = 0 \quad (6)$$

Boundary conditions relevant at time $t > 0$

Relevant conditions for the theoretical model represented with reaction scheme (I) that hold at time $t > 0$ are described with Eq. (7), i.e.:

$$t > 0; \Gamma(R) + \Gamma(Ox1) + \Gamma(Ox2) = \Gamma^*(R) \quad (7)$$

In addition, at $t > 0$, the change of surface concentration of each surface active redox species is directly related to the Faradaic current associated with its electron-transfer reaction. The equations that relate the time-dependent surface concentrations of all redox active species involved in mechanism (I) to the Faradaic current of both electron-transfer step, if kinetic constraints defined by Butler-Volmer formalism are assumed by both electron transfer steps, are given with Eqs. (8) and (9):

$$\frac{I_1}{n \cdot F \cdot A} = k_{s,1} \cdot e^{\alpha_1 \cdot \Phi_1} \cdot [\Gamma(R) - e^{-\Phi_1} \cdot \Gamma(Ox1)] \quad (8)$$

$$\frac{I_2}{n \cdot F \cdot A} = k_{s,2} \cdot e^{\alpha_2 \cdot \Phi_2} \cdot [\Gamma(Ox1) - e^{-\Phi_2} \cdot \Gamma(Ox2)] \quad (9)$$

In Eqs. (8) and (9), Φ_1 and Φ_2 stay for the dimensionless potentials defined as: $\Phi_1 = \frac{F}{RT}(E - E_1^0)$ and $\Phi_2 = \frac{F}{RT}(E - E_2^0)$, where E_1^0 and E_2^0 are the formal redox potentials of R(ads)/Ox1(ads) and Ox1(ads)/Ox2(ads) redox couples, respectively. In the definitions of Φ_1 and Φ_2 , R is the universal gas constant, T is thermodynamic temperature, n is number of electrons exchanged (n is set to 1 for both electron transfer steps), and F is the Faraday constant. With A is defined the effective area of working electrode, while the symbols α_1 and α_2 in Eqs. (8) and (9) stay for the electron transfer coefficients related to the first and the second electron transfer step, respectively. Within the framework of the surface-confined double-regenerative EC'EC'' mechanism examined here, the active surface of the working electrode may be treated as a boundary at which two distinct faradaic processes take place. The first faradaic current (I_1) originates from the interfacial electron-transfer reaction between the adsorbed redox couple R/Ox1, while the second faradaic current (I_2) is generated by the subsequent interfacial electron-transfer process involving the adsorbed species Ox1/Ox2. The contributions of irreversible regenerative reactions $Ox1(ads) + Y \rightarrow R(ads)$ and $Ox2(ads) + Y \rightarrow Ox1(ads)$ are incorporated into the differential Eq. (1) to (3). In contrast, the electrode kinetics are introduced exclusively through the interfacial boundary conditions expressed in Eqs. (8) and (9). The system of differential Eqs. (1)-(3) corresponding to the double regenerative two-step surface EC'EC'' mechanism was solved by exploring the mathematical protocols described in^{4,29}. A complete Mathcad working file developed for the calculation of square-wave voltammograms of considered EC'EC'' mechanism is freely available on demand. A detailed description of simulation protocols of an electrode mechanism in square-wave voltammetry implemented in the Mathcad commercial platform can be found in³⁰.

Parameters that affect simulated voltammetric patterns of the surface EC'EC'' mechanism

Before starting to analyze some of the most relevant theoretical results of the considered surface EC'EC'' mechanism, it is important to emphasize that the interpretation of the simulated responses is based on the behavior of the forward and backward components of the square-wave voltammetric current, but also the net SWV current components as well. At such reaction scheme, analysis of all current components provides a more transparent framework for assessing how individual kinetic parameters govern the entire voltammetric behavior. At this stage, it should be noted that the shape, position, and relative intensities of the voltammetric features are influenced by several experimental and intrinsic parameters, including the square-wave amplitude (E_{sw}), the potential increment (ΔE), and the temperature (T), which was fixed at 298.15 K in all simulations. Furthermore, the voltammetric response depends on the number of electrons exchanged in each electron-transfer step (n , n was set to 1 for both electron transfer steps) and on the electron-transfer coefficients α_1 and α_2 , both of which were set to 0.5 throughout this study. The dimensionless currents in SWV are normalized according to $\Psi = I/nFAf\Gamma^*(R)$, where I is the real current, n is the number of electrons transferred (equal to 1 for both electron transfer steps), F is the Faraday constant, A is the electrode surface area, $\Gamma^*(R)$ is the initial surface concentration of the

redox-active species R, and f is the square-wave frequency. Since the present analysis is formulated for a surface-confined redox system, mass-transport effects do not contribute to the voltammetric response. Consequently, the kinetics of both electron-transfer steps are described solely by their dimensionless surface kinetic parameters ($K1$ and $K2$), defined as:

$$K1 = k_{s,1}/f; \text{ and} \quad (10)$$

$$K2 = k_{s,2}/f \quad (11)$$

where $k_{s,1}$ and $k_{s,2}$ are the standard heterogeneous electron-transfer rate constants of the first and second electron-transfer steps, respectively, and f is the square-wave frequency. These dimensionless parameters express the intrinsic electron-transfer kinetics relative to the time scale imposed by the square-wave excitation and thus fully characterize the interfacial charge-transfer dynamics in surface-immobilized systems.

In addition to electron-transfer kinetics, the simulation algorithm of EC'EC'' mechanism incorporates two regenerative (catalytic) steps, which are quantified by the dimensionless catalytic parameters $K_{chem,1}$ and $K_{chem,2}$, defined as:

$$K_{chem,1} = k_{c,1}/f; \text{ and} \quad (12)$$

$$K_{chem,2} = k_{c,2}/f \quad (13)$$

In Eqs. (12) and (13), $k_{c,1}$ and $k_{c,2}$ denote the effective catalytic rate constants of the first and second regenerative reactions, respectively, defined as

$$k_{c,1} = k'_{c,1} \cdot c(Y); \text{ and} \quad (14)$$

$$k_{c,2} = k'_{c,2} \cdot c(Y); \quad (15)$$

where $k'_{c,1}$ and $k'_{c,2}$ are the intrinsic catalytic rate constants, and $c(Y)$ represents the molar concentration of the catalytic agent Y, assumed to be present in large excess in voltammetric cell.

In majority of the simulations, a peak separation of 300 mV was imposed to ensure clear discrimination between the voltammetric features associated with each electron-transfer step. Although some of the simulated conditions may be challenging to realize experimentally, their theoretical analysis provides valuable insight into the intrinsic behavior of the EC'EC'' mechanism as a whole. The primary objective of the theoretical investigation is to elucidate catalytic effects by systematically varying $K_{chem,1}$ and $K_{chem,2}$, and to identify characteristic voltammetric patterns that facilitate mechanistic interpretation and kinetic evaluation of this complex surface-confined electrode process. Throughout the simulations, all parameters were kept constant except for the single parameter under investigation, enabling an unambiguous assessment of its specific influence on the voltammetric responses. All figures in the main text are given in 3D form for a better visualization, while the corresponding 2D graphs are given in the Supplementary material.

Results and discussion

The simulated square-wave voltammograms displayed in Fig. 1 illustrate the progressive influence of the first catalytic regeneration step on the electrochemical response of a surface-confined EC'EC'' mechanism, in scenario where the rate regenerative constant of second catalytic step remains negligible. In the particular case shown as pattern (a) in Fig. 1A, the kinetic rate constants of both regenerative (catalytic) steps are negligible. Under these conditions, neither of the chemical regenerative reactions contributes measurably to the overall electrochemical response. Consequently, the voltammetric outputs are manifested as nearly symmetrical pairs of sharp

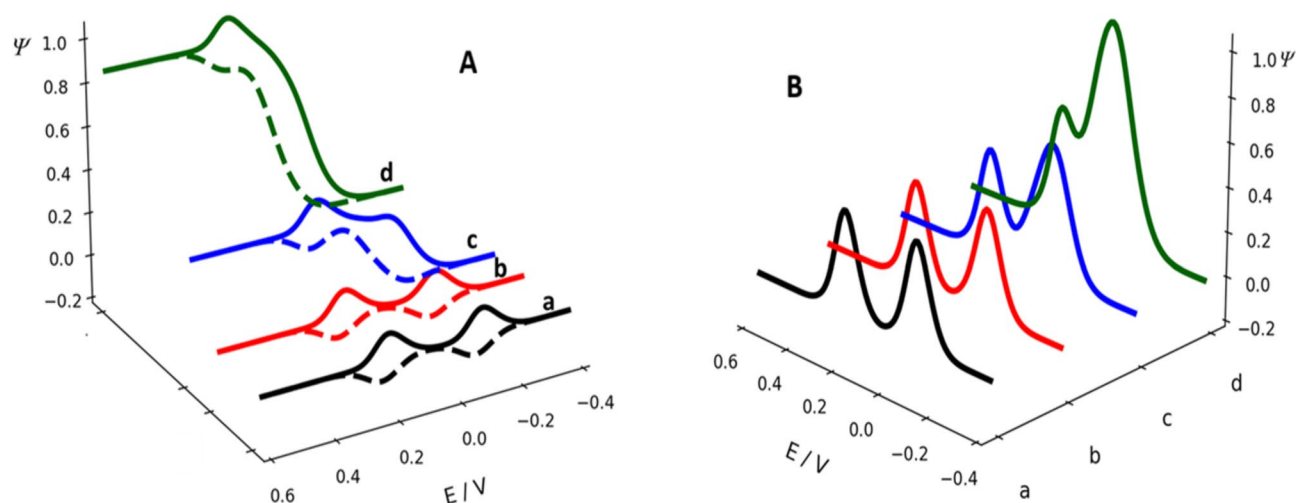


Fig. 1 (A) Simulated forward (solid lines) and backward (dashed lines) square-wave voltammetric current components for a surface-confined double-regenerative EC'EC'' mechanism under conditions where the second catalytic (regenerative) step is kinetically negligible. The dimensionless catalytic parameter of the second chemical step was fixed at $K_{chem,2}=0.0001$ for all curves, while the catalytic parameter of the first regenerative step was varied as $K_{chem,1}=0.0001$ (a), 0.1 (b), 1.0 (c), and 2.5 (d). The dimensionless electron-transfer rate parameters for both electron-transfer steps were identical and set to $K_1=K_2=0.398$. Equal electron-transfer coefficients for both electron transfers were assumed, set to $\alpha_1=\alpha_2=0.5$ in all simulations. Other simulation parameters were: square-wave amplitude $E_{sw}=50$ mV, potential step $dE=10$ mV, and temperature $T=298.15$ K. 300 mV is the potential separation defined between both electron transfer steps. Panel (B) shows the corresponding net square-wave current components.

forward and backward peaks, characteristic of a quasireversible surface-confined redox process⁴. In the absence of catalytic regeneration, the EC'EC'' mechanism effectively collapses into a purely sequential two-step electron-transfer pathway (curve **a** in Fig. 1A), and the entire system behavior converges to that of a surface-confined EE mechanism³¹. In the voltammograms displayed Fig. 1A, which show the separated forward and backward current components, an increase in the rate constant of the first catalytic step from (a) to (d) leads to a pronounced amplification and distortion of the forward current component, whereas the backward component is progressively suppressed. This behavior reflects efficient catalytic regeneration of the electroactive species following the first electron-transfer step, which enhances the anodic current component while simultaneously depleting the population of the reversibly transformable surface species during the reverse half-cycle. As a consequence, the degree of asymmetry between the forward and backward components increases monotonically with increasing catalytic strength, providing a clear diagnostic signature of surface-confined EC' behavior^{1-4,14,18}.

In the corresponding net square-wave voltammograms shown in Fig. 1B, these effects manifest as a systematic increase in peak current accompanied by peak broadening and a slight shift in peak position as the first catalytic rate constant increases from (a) to (d). The enhanced peak currents directly originate from the dominance of the forward component over the backward one, while the preservation of a single main peak indicates that the second catalytic step does not contribute measurably to the overall response. Importantly, the smooth evolution of the net peak shape, without the appearance of additional features, confirms that the observed voltammetric changes are governed exclusively by the kinetics of the first regenerative step and are characteristic of a surface-confined single-catalytic EC' regime embedded within the formal EC'EC'' framework.

In the second scenario elaborated in this part of the work (Fig. 2A and B), the kinetic constant of the first regenerative step is kept negligible ($K_{chem,1}=0.0001$), while only the catalytic parameter of the second chemical step is varied ($K_{chem,2}=0.0001$ (a), 0.25 (b), 0.75 (c), 1.25 (d), and 2 (e)). Although such conditions may be difficult to realize experimentally, since both catalytic pathways are typically coupled, this isolated variation provides mechanistic clarity regarding the role of the second catalytic reaction. Under these circumstances, the

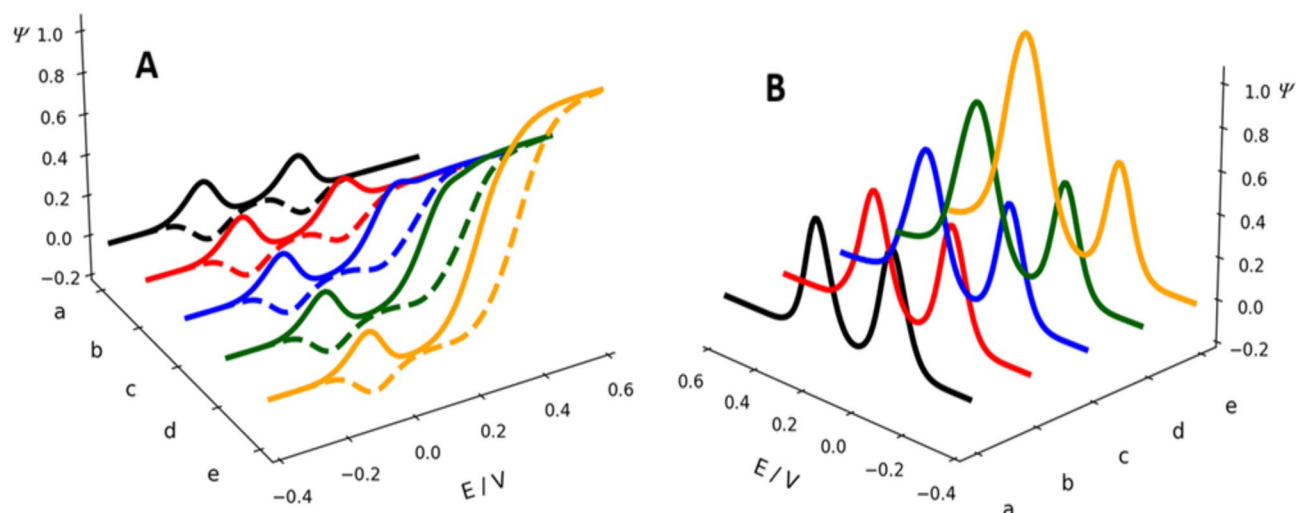


Fig. 2 (A) Simulated forward (solid lines) and backward (dashed lines) square-wave voltammetric current components for a surface double-regenerative EC'EC'' mechanism under conditions where the first catalytic (regenerative) step is kinetically negligible. The dimensionless catalytic parameter of the first regenerative step was fixed at $K_{chem,1} = 0.0001$ for all curves, while the catalytic parameter of the second regenerative step was varied as $K_{chem,2} = 0.0001$ (a), 0.25 (b), 0.75 (c), 1.25 (d) and 2 (e). Panel (B) shows the corresponding net square-wave current components. Other simulation parameters were identical as those in Fig. 1.

EC'EC'' scheme effectively converges toward a surface EEC' mechanism, in agreement with the theoretical treatment reported previously³².

In Fig. 2A, curve a, where magnitude of $K_{chem,2}$ is negligible, forward and backward peaks exhibit nearly symmetrical features, corresponding to a surface-confined two-step EE process³¹, since neither regenerative step significantly perturbs the redox equilibrium. As $K_{chem,2}$ increases (patterns b–e), pronounced asymmetry develops selectively in the second redox couple. The forward component associated with the second electron-transfer step becomes progressively enhanced, while the corresponding backward peak diminishes in intensity and shifts towards anodic region, reflecting depletion of the intermediate species through catalytic regeneration. This behavior is characteristic signature of an EC'-type catalytic feedback^{1–4,14,18}. In such scenario, the chemical step following the second electron transfer continuously regenerates the electroactive form, while amplifying the forward current and suppressing the reverse current component. In contrast, the first redox pair remains largely unaffected, confirming that the first catalytic pathway does not contribute under the chosen conditions.

The net SWV responses shown in Fig. 2B nicely illustrate the catalytic transformation. For small $K_{chem,2}$ (a), the net voltammogram consists of two relatively sharp and well-defined peaks typical of a two-step surface EE mechanism. With increasing $K_{chem,2}$ (curves b–e), the second peak undergoes substantial amplification and distortion, evolving into a broader and more intense feature. The first peak remains nearly invariant throughout the series, again confirming that only the second redox step participates in catalytic regeneration. At higher $K_{chem,2}$ values (curves d and e), the second peak exhibits pronounced catalytic enhancement and certain potential displacement, reflecting the increasing dominance of the chemical feedback over the intrinsic electron-transfer kinetics.

In general, the curves presented in Fig. 2A–B demonstrate that selective acceleration of the second regenerative step produces classical EC' signatures localized at the second electron-transfer process. The mechanistic consequence is a functional reduction of the original EC'EC'' scheme to a surface EEC' pathway³², where the first step behaves as a simple reversible surface electron transfer, while the second peak displays catalytic amplification governed by the rate of second regenerative step, expressed via the magnitude of the dimensionless chemical regenerative parameter $K_{chem,2}$.

In the third elaborated scenario (Fig. 3A and B), both electron-transfer steps are very fast ($K_1 = K_2 = 7.08$), so that the interfacial redox conversion occurs predominantly at the very beginning of each square-wave potential pulse. As a result, the forward and backward current components in Fig. 3A become strongly shifted apart from each other, reflecting the fact that the system responds almost instantaneously to the potential perturbation in each half-cycle and the two directional components no longer substantially overlap. At the same time, the detected currents are much lower than in the quasireversible cases (Figs. 1 and 2), because SWV samples the current near the end of each pulse: when electron transfer is very fast, most of the surface-confined electroactive population has already been converted early in the pulse, leaving only a small residual amount to contribute to the measured current at the sampling time. This is a hallmark of surface-confined systems under fast kinetics: high conversion efficiency early in the pulse, but reduced signal at the end of the pulse where current is evaluated^{4,33}.

A key outcome in this regime is that both electron-transfer steps become markedly more susceptible to regenerative feedback, because the catalytic steps can “recycle” the electroactive forms within the same pulse much earlier than in quasireversible regimes. When $K_{chem,1} = K_{chem,2}$ is very small (curve **a** in Fig. 3B), the response primarily reflects surface EE behavior with very fast electron transfer steps, and the net SWV in Fig. 3B exhibits the characteristic splitting phenomenon (two-peak structure at each peak), which arises when the forward and backward peaks are sufficiently separated, so the net response resolves them as distinct features. Upon increasing catalytic strength from (a) to (e) ($0.0001 \rightarrow 0.05$), the regenerative steps increasingly return the already converted surface species back to its electroactive form within the time frame of the square-wave pulses, enabling repeated electrochemical turnover (“multiple reuse” of the surface-confined redox sites). This so-called “intra-pulse recycling” progressively enhances the catalytic contribution to the measured current, despite the inherently low late-pulse signal expected for very fast electron transfer⁴.

The net responses in Fig. 3B clearly demonstrate another diagnostic feature of the EC’EC” surface double-regenerative mechanism under very fast kinetics the splitting gradually diminishes and ultimately disappears at higher catalytic rates (at $K_{chem} > 0.075$ under conditions defined for Fig. 3). Mechanistically, this occurs because strong catalytic recycling smooths the temporal asymmetry between the two half-cycles. That is, the regeneration reaction replenishes electroactive material in a manner that sustains current throughout the pulse sequence and

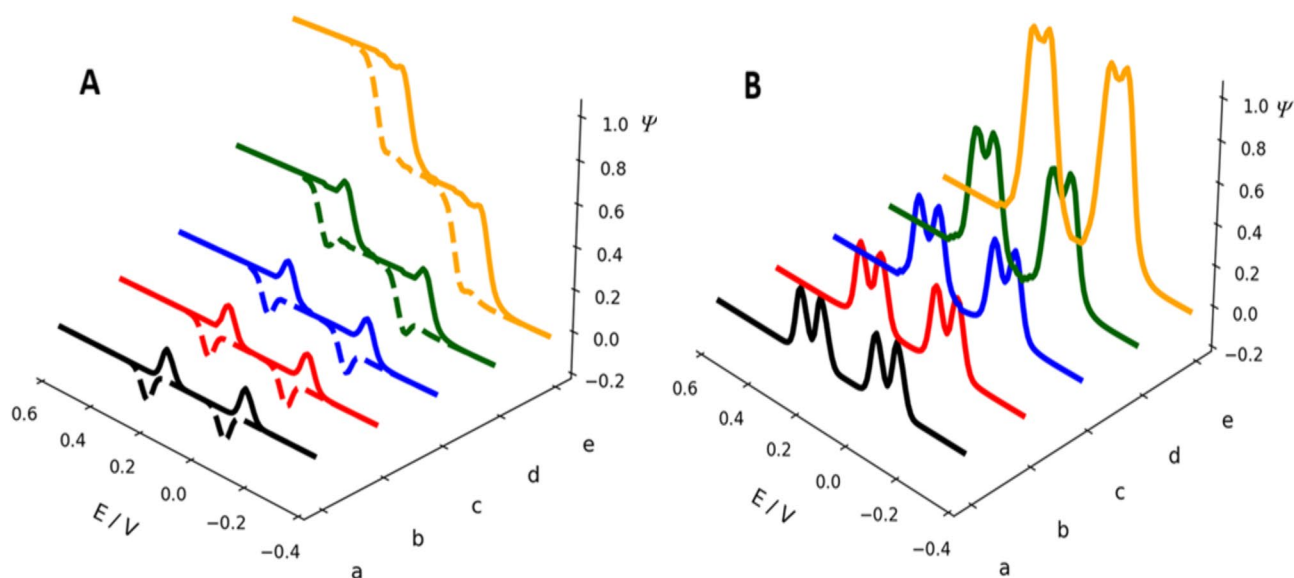


Fig. 3 (A) Simulated forward (solid lines) and backward (dashed lines) square-wave voltammetric current components for a surface-confined double-regenerative EC’EC” mechanism under conditions of fast and equal electron transfer rates of both steps featuring $K_1 = K_2 = 7.08$. The dimensionless catalytic parameter of the first and second chemical step were equal and fixed at $K_{chem,1} = K_{chem,2} = 0.0001$ (a); 0.001 (b); 0.005 (c); 0.01 (d) and 0.05 (e). Panel (B) shows the corresponding net square-wave current components. Other simulation parameters were identical as those in Fig. 1.

effectively merges what would otherwise appear as two separated contributions in the net signal³⁴. Thus, at larger $K_{chem,1} = K_{chem,2}$ (patterns d–e in Fig. 3B), the net response evolves from a split, surface EE-like pattern into a more unified catalytic feature, consistent with dominant regenerative control over the pulse-to-pulse population dynamics.

In comparison with the quasireversible scenarios in Figs. 1 and 2, where catalytic effects develop more gradually because electron transfer itself limits how quickly the redox population can be cycled, the present fast-electron transfer regime in Fig. 3 amplifies the influence of the chemical steps. In such scenario, the regeneration acts earlier within each pulse and therefore more effectively increases the number of electrochemical turnovers per cycle. Consequently, the combination of (i) low baseline currents due to early pulse conversion, (ii) strong separation of forward/backward components, and (iii) catalytic suppression of splitting of net SWV peaks at higher K_{chem} values constitutes a distinctive voltammetric signature of a surface-confined EC'EC'' mechanism operating with very fast electron transfer at both steps.

In the so-called “mixed-kinetic scenario” of surface EC'EC'' double regenerative mechanism presented in Fig. 4, characterized with $K_1 = 0.631$ (moderate/quasireversible electron transfer), and $K_2 = 4.47$ (fast electron transfer), equal catalytic strengths applied to both regenerative steps provide a particularly instructive contrast in how EC' signatures emerge at each redox transformation. In Fig. 4A, the features of forward and backward components show that the catalytic influence at the second electron transfer step becomes apparent already at catalytic rates that are roughly one order of magnitude lower than those required to produce a comparable distortion at the first step. This higher sensitivity of the second peak is a direct consequence of its fast electron-transfer kinetics. Namely, because the second electron transfer step converts the surface population early within each SW pulse, the catalytic regeneration can recycle the electroactive form within the same pulse window more effectively, thereby amplifying the forward component and suppressing the backward one at relatively small K_{chem} values. In contrast, the first electron-transfer step remains partially limited by its relatively slow kinetics. As a result, a stronger regenerative feedback is required before the balance between the forward and backward current components is sufficiently perturbed to produce the characteristic asymmetry associated with regenerative mechanisms.

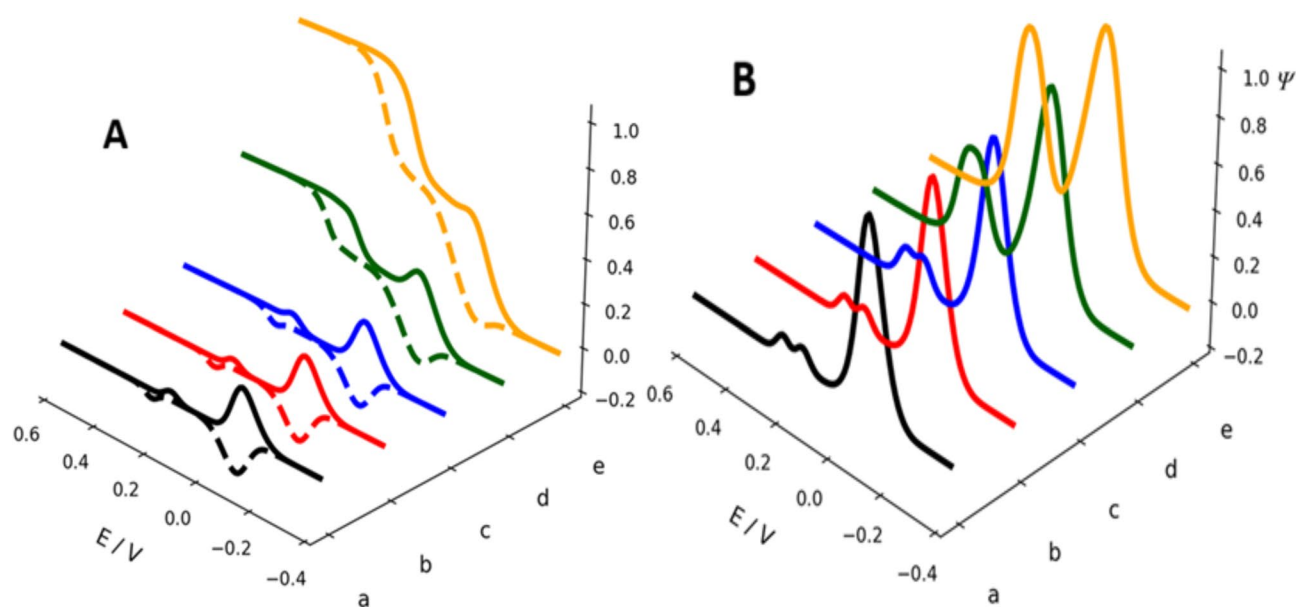


Fig. 4 (A) Simulated forward (solid lines) and backward (dashed lines) square-wave voltammetric current components for a surface-confined double-regenerative EC'EC'' mechanism under conditions of “mixed: electron transfer rates characterized with $K_1 = 0.631$, and $K_2 = 4.47$. The dimensionless catalytic parameter of the first and second chemical step were equal and fixed at $K_{chem,1} = K_{chem,2} = 0.0001$ (a); 0.01 (b); 0.1 (c); 0.5 (d) and 1.0 (e). Panel (B) shows the corresponding net square-wave current components. Other simulation parameters were identical as those in Fig. 1.

The net responses in Fig. 4B further highlight much obvious the kinetic interplay. In the baseline case of negligible catalysis (curve a), the second peak (governed by fast electron transfer) displays the characteristic splitting phenomenon, but its net magnitude is more than an order of magnitude smaller than that of the first peak. This reduced signal of second peak is consistent with surface SWV under fast kinetics, where most of the electroactive material is transformed near the beginning of each pulse, leaving only a small fraction to contribute at the end-of-pulse sampling time. As catalytic rates are increased, however, this initially “weak” second peak becomes disproportionately amplified because regeneration repopulates the electroactive state earlier and repeatedly during the pulse sequence, while effectively enabling multiple turnovers of the same surface-confined material. Consequently, the splitting feature progressively diminishes and ultimately disappears at higher catalytic strengths, evolving into a single, catalytically enhanced net peak. At sufficiently large catalytic rates (curve e in Fig. 4B), both peaks become single and approach comparable intensities, despite their very different baseline behaviors (curves a in Fig. 4A-B). This convergence indicates that strong regeneration compensates for the kinetic “late-sampling” penalty of fast electron transfer step by sustaining measurable current throughout the pulse, while simultaneously producing the classical EC' pattern (forward dominance and backward suppression) at both redox steps.

In general, Fig. 4A and B demonstrate that, even when catalytic rate parameters are equal, i.e., $K_{\text{chem},1} = K_{\text{chem},2}$, the apparent catalytic onset and the evolution of peak morphology are strongly modulated by the intrinsic electron transfer kinetics of each step. Specifically, the faster (in this case the second electron transfer) step exhibits catalytic signatures earlier (at lower K_{chem}) and transitions from split to single-peak behavior. In current scenario, the quasireversible (first electron transfer) step requires stronger catalysis to reach a similarly expressed EC' regime. This “two-speed” system elaborated in Fig. 4 therefore serves as a useful mechanistic playground for disentangling how regenerative feedback couples to surface-confined electron transfer across moderate versus fast kinetic limits.

When both electron-transfer steps of the surface EC'EC'' scheme occur at the same formal potential, the voltammetric response becomes intrinsically degenerate, because the two redox transformations are no longer spectroscopically/voltammetrically separable in the potential domain. Under the present conditions ($K_1 = 0.631$, $K_2 = 4.47$, and both K_{chem} varied from 10^{-4} to the higher values assigned for patterns a–e), Fig. 5A shows that increasing catalytic strength produces the expected EC'-type evolution. In such scenario, the forward component grows while the backward component is suppressed, and the response progressively becomes more asymmetric as regeneration becomes more efficient. However, because both electron transfers share the same potential, the catalytic feedback acting after each step cannot generate two distinct peak systems; instead, their contributions collapse into a single composite feature whose morphology and forward/backward imbalance are essentially indistinguishable from a one-step surface EC' mechanism with appropriately chosen effective kinetic parameters.

This is precisely why, in such a scenario, it is almost impossible (based solely on SWV shape alone) to decide uniquely whether the underlying mechanism is truly EC'EC'' (two electron transfers with two regenerative steps) or a simpler single-step EC' process. Any increase in K_{chem} simply drives the system more deeply into the catalytic regime. However, the “fingerprints” that normally allow EC'EC'' recognition (peak separation), differential catalytic onset at two peaks, splitting behavior localized to one step, or selective distortion of one redox couple, are all lost when the potentials coincide. In other words, the mechanism becomes structurally unidentifiable from a single voltammetric experiment because different mechanistic models can reproduce the same forward/backward decomposition and the same net response.

Nevertheless, mechanistic discrimination is still possible in principle, but it requires additional experimental means that reintroduce separability between the two electron transfers. The most direct strategy is to employ conditions or complementary techniques that can resolve the steps when the two redox potentials are *not* coincident, particularly in the challenging case of inverted redox potentials (where the order of the formal potentials is reversed). In practice, this requires combining square-wave voltammetry with complementary experimental approaches. One possibility is to apply perturbations that selectively shift one formal potential relative to the other. Such perturbations may include changes in pH, complexation via specific ligand binding of one of the

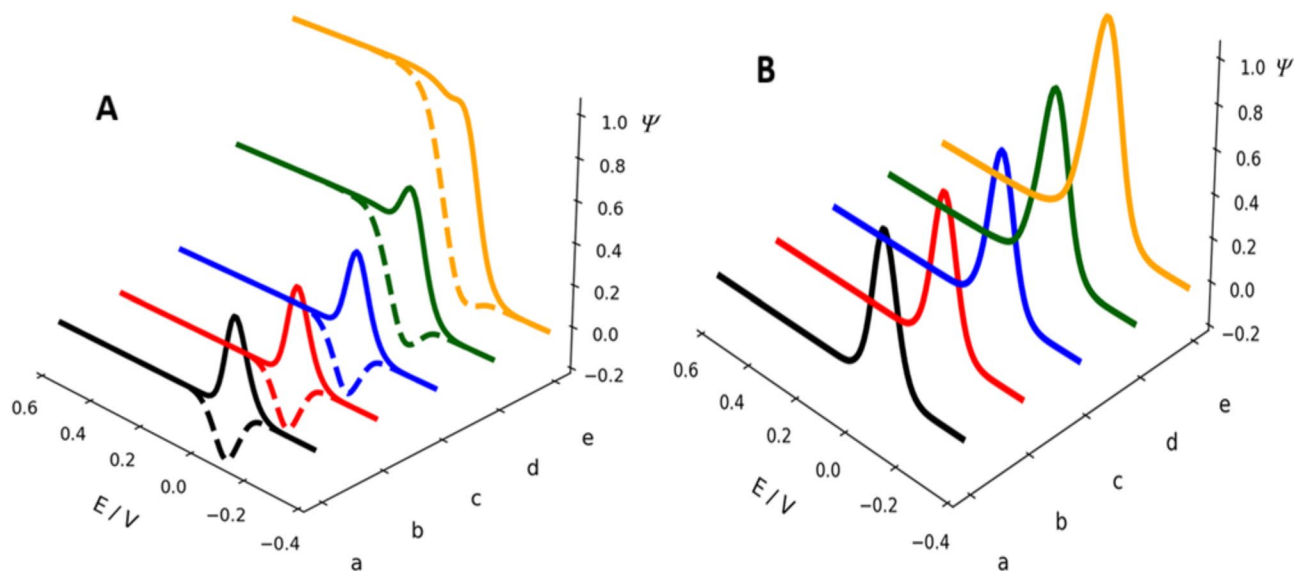


Fig. 5 (A) Simulated forward (solid lines) and backward (dashed lines) square-wave voltammetric current components for a surface-confined double-regenerative EC'EC'' mechanism under conditions of “mixed: electron transfer rates characterized with $K_1=0.631$, and $K_2=4.47$, when both processes occur at same potential. The dimensionless catalytic parameter of the first and second chemical step were equal and fixed at $K_{chem,1}=K_{chem,2}=0.0001$ (a); 0.01 (b); 0.1 (c); 0.5 (d) and 1.0 (e). Panel (B) shows the corresponding net square-wave current components. Other simulation parameters were identical as those in Fig. 1.

participants, or other specific interactions affecting only one redox center. Another strategy is to perform kinetic fingerprinting over a broad range of square-wave frequencies. This approach makes it possible to eventually evaluate whether the experimental response can be described by a single EC' time scale or whether two coupled electron-transfer time scales are required. Finally, other techniques, such as spectroelectrochemistry and electrochemical impedance spectroscopy may provide additional evidence for the existence of two distinct redox states, even when the square-wave voltammetric response collapses into a single peak. In general, the scenario in which both electron transfers occur at same-potential is a useful theoretical limit, since it demonstrates that peak-shape voltammetric diagnostics alone are insufficient for unequivocal mechanism assignment.

Practical guidelines for kinetic evaluations

Reliable extraction of kinetic parameters from protein-film square-wave voltammetry is one of the principal motivations for developing theoretical models of complex surface-confined catalytic mechanisms. For many biologically important lipophilic enzymes and redox proteins,^{9–12} quantitative knowledge of the intrinsic heterogeneous electron-transfer rate constants, electron-transfer coefficients and catalytic regeneration rates provides direct insight into enzymatic turnover,³ catalytic efficiency, electron-transfer pathways and biological function^{5–12}. These parameters are particularly valuable when comparing native and mutated proteins, investigating inhibitor binding, evaluating catalytic activity under different physiological conditions, or designing biomimetic catalytic interfaces^{3,9,10}. Consequently, successful application of the present theoretical framework requires a systematic experimental protocol in which the kinetic parameters are determined sequentially rather than simultaneously.

As a first step, it is highly advisable to characterize the electrochemical properties of the investigated protein in the complete absence of the regenerative substrate “Y”. Under these conditions, both catalytic reactions are suppressed (i.e., $K_{chem,1} \approx K_{chem,2} \approx 0$), and the investigated mechanism effectively reduces to a simple two-step surface-confined EE mechanism³¹. Such experimental conditions enable independent determination of the intrinsic electron-transfer parameters governing both electrode processes without interference from catalytic feedback.

Whenever both redox transformations are sufficiently separated in the potential domain (at least for 150 mV), the characteristic *splitting of net square-wave voltammetric peaks*³³ may be exploited for quantitative estimation of the heterogeneous electron-transfer rate constants $k_{s,1}$ and $k_{s,2}$, together with the corresponding electron-transfer coefficients α_1 and α_2 . The theoretical basis of this methodology has been extensively described for surface-confined systems³³ and later comprehensively summarized in the monograph⁴. These procedures remain among the most robust experimental tools available for characterization of surface-confined quasireversible electron-transfer processes.

An attractive alternative for evaluation of the electron-transfer parameters is based on the analysis of half-peak widths of the individual “net” square-wave voltammetric responses³³. This methodology, originally proposed for surface-confined protein-film voltammetry, enables simultaneous estimation of the heterogeneous rate constants and transfer coefficients through analysis of the characteristic broadening of square-wave peaks under quasireversible conditions. The approach has been demonstrated to work for various surface-confined redox systems³⁵ and represents an experimentally straightforward route whenever sufficiently well-resolved peaks are available.

A third possibility consists of complete numerical fitting of normalized square-wave voltammograms using the theoretical model elaborated in the present work, with full Mathcad simulation protocol available upon request from the authors. Instead of relying on individual voltammetric characteristics, such as peak splitting or half-peak widths, the complete experimental (normalized) response is fitted by iterative adjustment of the model parameters until optimal agreement between theoretical and experimental voltammograms is achieved. Similar numerical fitting strategies have previously been successfully employed in square-wave voltammetry for analysis of complex mechanisms^{11,20,30} and provide an especially valuable approach when several kinetic parameters simultaneously influence the voltammetric response.

Once the intrinsic electron-transfer parameters of both redox transformations have been established, the regenerative substrate “Y” may gradually be introduced into the electrochemical cell. Under these conditions, the previously determined values of $k_{s,1}$ and $k_{s,2}$, α_1 and α_2 remain fixed, while only the catalytic parameters are varied during subsequent analysis. The most rigorous approach again consists of complete numerical fitting of the theoretical model³⁶, now using $k_{c,1}$ and $k_{c,2}$ as the only adjustable kinetic variables. Such a sequential fitting strategy considerably reduces parameter correlation and substantially improves the reliability of the obtained catalytic constants.

Alternatively, the rates of both regenerative chemical reactions may be estimated experimentally from the limiting catalytic currents recorded at increasing concentrations of the regenerative substrate “Y”. Since the corresponding effective catalytic parameters are directly proportional to substrate concentration (i.e. $k_c = k'_c \cdot c(Y)$), plots of limiting peak currents versus the concentration of “Y” provide an independent route for determination of the intrinsic catalytic rate constants¹⁸. Similar concentration-dependent methodologies have previously been developed for surface catalytic mechanisms in square-wave voltammetry³⁷ and represent an experimentally convenient strategy, whenever reliable concentration series can be obtained.

Finally, it should be emphasized that extraction of kinetic parameters from complex surface catalytic mechanisms should never rely on fitting all unknown quantities simultaneously. Such an approach frequently leads to strong parameter correlation and non-unique solutions, particularly in multistep catalytic systems such as the present surface EC'EC" mechanism. Instead, sequential determination of electron-transfer parameters followed by catalytic rate evaluation offers a substantially more robust and physically meaningful strategy.

As a practical step-by-step workflow, an experimental kinetic analysis may begin by recording a series of SW voltammograms of the surface-confined redox protein in the absence of the regenerative substrate, preferably over a suitable range of SW frequencies. These measurements should first be used to estimate $k_{s,1}$, $k_{s,2}$, α_1 and α_2 from peak splitting, half-peak widths, or numerical fitting of the normalized voltammograms. In the next step, a concentration series of the regenerative substrate is introduced and the corresponding catalytic SW voltammograms are recorded under otherwise identical experimental conditions. The catalytic rate constants $k_{c,1}$ and $k_{c,2}$ can then be obtained by fitting the concentration-dependent responses with the present theoretical model. As a

final validation step, the resulting parameter set should be used to simulate voltammograms recorded at additional substrate concentrations and/or SW frequencies that were not employed in the initial fitting procedure.

The theoretical fingerprints established in the present work, together with the available analytical methodologies for square-wave protein-film voltammetry, provide a comprehensive framework for quantitative characterization of a broad family of biologically relevant redox proteins and enzymes operating through sequential double-regenerative electron-transfer pathways.

Conclusions

In this work, the surface-confined EC'EC'' double-regenerative electrode mechanism has been theoretically analyzed for the first time under square-wave protein-film voltammetric conditions. By exploring a wide range of kinetic regimes for both electron-transfer steps and their associated chemical regeneration reactions, several characteristic scenarios have been identified, elucidating how the interplay between electron-transfer kinetics and catalytic feedback governs the voltammetric responses. Particular emphasis was placed on the analysis of forward and backward, as well as the net SW current components, which all together proved essential for elucidating subtle mechanistic signatures that are often obscured in net square-wave voltammograms.

The results demonstrate that, depending on the relative magnitudes of the chemical regenerative parameters and the intrinsic electron-transfer rates, the EC'EC'' mechanism may effectively converge toward simpler limiting cases. Specifically, when both regenerative steps are negligible, the response collapses to that of a surface-confined EE mechanism. Selective acceleration of only one regenerative step leads to behavior characteristic of surface EC'E or EEC' pathways, whereas strong regeneration combined with a single dominant electron-transfer step produces voltammetric features indistinguishable from a simple surface EC' mechanism. Special attention was devoted to scenarios involving fast electron transfer at one or both steps, where pronounced separation of forward and backward components and the appearance of peak splitting phenomena were observed. It was shown that increasing catalytic strength can suppress splitting in the net response by enabling early and repeated regeneration of electroactive material within the square-wave pulses. In systems where both electron-transfer steps occur at the same formal potential, or in the particularly challenging case of inverted redox potentials, the voltammetric signatures of the EC'EC'' mechanism become degenerate with those of simpler EC' systems. Under such conditions, it is practically impossible to unambiguously identify the underlying double-regenerative pathway from square-wave voltammetry alone, and additional complementary experimental techniques are required for reliable mechanistic diagnosis.

From a kinetic perspective, access to the electron-transfer parameters of both steps can be achieved using established methodologies, such as quasireversible maximum analysis or net-peak splitting diagnostics, following procedures previously elaborated in the literature⁴. Likewise, the rates of regenerative chemical steps may be extracted using protocols developed for surface catalytic mechanisms in square-wave voltammetry^{18,33}. Nevertheless, systems with overlapping or inverted redox potentials remain among the most challenging cases, reinforcing the need for multi-technique approaches when investigating complex catalytic redox networks³⁸.

At this stage it is worth to underline that frequency analysis is often employed in square-wave voltammetry as a valuable tool for extracting kinetic parameters of electrode mechanisms coupled with regenerative chemical reactions. However, some caution is required for the complex surface EC'EC'' mechanism, since variation of the SW frequency simultaneously affects the kinetics of both electron-transfer steps as well as the dimensionless kinetic parameters associated with both regenerative chemical reactions. Consequently, frequency-based analysis may lead to erroneous kinetic estimations, particularly when both electron-transfer steps operate within the slow-to-moderate kinetic regime³⁹. Under such conditions, alternative strategies, such as those based on variation of the SW amplitude or by using Electrochemical Faradaic Spectroscopy,³⁸ may provide a more reliable means of assessing the kinetics of the individual electron-transfer steps. In contrast, the kinetics of the two regenerative

chemical reactions can be evaluated by systematically varying the concentration of the regenerative substrate, following the methodology described elsewhere^{18,40}.

In the end, it is worth mentioning that the surface-confined double regenerative EC'EC" mechanism elaborated in this work can be seen as a versatile and informative model system. It is a useful theoretical platform for probing the fundamental relationships between electron transfer, chemical regeneration, and voltammetric features in square-wave voltammetry. Owing to its direct relevance to the electrochemical behavior of many lipophilic proteins, redox enzymes, and biomimetic systems, the present framework is particularly well suited for protein-film voltammetry, and also for various surface-confined biochemical systems. To facilitate further exploration and independent validation, the complete Mathcad simulation protocol developed in this work is available for free on demand, and also included in the Supplementary Material. It offers a flexible platform for studying a broad spectrum of kinetic and mechanistic aspects of this complex yet highly relevant electrode process.

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Data availability All data supporting the findings of this study, including the original Mathcad simulation file, are available from the authors upon request. In addition, entire Mathcad simulation protocol is available In Supplementary Material in this work and also on: <https://eprints.ugd.edu.mk/38192>.

Declarations

Competing interests The authors declare no competing interests.

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Authors and Affiliations

Milkica Janeva¹ · Pavlinka Kokoskarova¹ · Rubin Gulaboski¹ 

✉ Rubin Gulaboski
rubin.gulaboski@ugd.edu.mk

¹ Faculty of Medical Sciences, Goce Delcev University, Stip, Republic of Macedonia