Time-Independent Methodology to Access Michaelis-Menten Constant by Exploring Electrochemical-Catalytic Mechanism in Protein-Film Cyclic Staircase Voltammetry

Rubin Gulaboski,* Pavlinka Kokoskarova, Sofija Petkovska

Faculty of Medical Sciences, „Goce Delcev" University, Stip, Macedonia
* Corresponding author's e-mail address: rubin.gulaboski@ugd.edu.mk

Abstract: Protein-film voltammetry is recognized as a very efficient tool in mechanistic enzymology, but it is also seen as a relevant approach to gain thermodynamic and kinetic information related to the redox chemistry of many enzymes. This technique requires a small amount of redox enzyme, whose molecules form monomolecular film on the working electrode surface. In this paper we present a simple and time-independent cyclo-voltammetric method for the determination of kinetics of the chemical step of an electrochemical-catalytic (EC') mechanism in protein-film scenario. Theoretical results of a surface EC' mechanism show that the limiting cyclo-voltammetric catalytic current, measured at large overpotentials, depends solely on the rate of the chemical regenerative reaction. At large overpotentials, the limiting current of the steady-state cyclic voltammograms is independent on all kinetics and thermodynamic parameters related to the electrode reaction of adsorbed enzyme. The approach proposed relies on the dependence of the magnitude of limiting current of the experimental cyclic steady-state voltammograms as a function of the substrate concentration.

Keywords: surface EC' mechanism, catalytic rate constant, cyclic voltammograms, enzymatic reactions, protein-film voltammetry.

INTRODUCTION

UNDERSTANDING the mechanism of electron-exchange between redox enzymes and their substrates is, indeed, one of the most fundamental aspects in biological chemistry. Hence, great efforts have been made to evaluate the thermodynamics and kinetics related to redox features of many important enzymes.[1–12] Whenever there is an electron exchange between an enzyme and a given substrate, voltammetry is seen as a very suitable technique to access thermodynamic and kinetic parameters related to the enzyme-substrate interactions. Indeed, when it is about enzymes electrochemistry, application of a given voltammetric technique is not always an easy task. Main troubles are seen in huge energetic barrier to reach enzymes redox active site(s).[4] The presence of big insulating parts in enzyme structures usually impedes significantly the electron exchange between working electrode and the enzyme's redox-active centre. In the end of the 20th century, a new voltammetric approach, the "protein-film

voltammetry", emerged as a promising tool to study the electrochemical features of lipophilic redox enzymes.[5] This technique is seen as an efficient approach for providing access to important information about enzymes redox chemistry.[4–13] When a given enzyme is attached on working electrode surface (mainly by self-assembling), then its redox features can be analyzed voltammetrically, if externally-controlled potential is applied to the enzyme-modified electrode. By applying cyclic voltammetry (CV) or square-wave voltammetry (SWV) as a working technique, valuable information about enzymes redox chemistry can be evaluated. The experimental data obtained in protein-film voltammetric set-up are analyzed mainly in form of plots of the mid-peak potentials or the peak currents against the scan rates applied. In this way, one can evaluate thermodynamic and kinetic data relevant to the biochemical functions of many redox enzymes.[2–13] Among the protein-film mechanisms elaborated with cyclic voltammetry, the surface electrochemical-catalytic mechanism (or simply "surface EC' mechanism") is one of the most

comprehensively studied.[2,4–8,10,13–22] This mechanism is quite relevant from physiological point of view, since it mimics real aspects of many enzyme-substrate systems.[3–5,8,13] The surface EC' mechanism considers an electron transfer step (E) between the working electrode and a redox enzyme firmly adsorbed at the electrode surface, which is coupled to a subsequent chemical reaction (C'). The chemical step of the surface EC' mechanism contributes to regeneration of the starting enzyme redox form on the electrode surface. This is achieved via a subsequent redox reaction between the final product of the enzymatic electrode reaction and a given substrate that is present dissolved in the electrochemical cell. Many relevant aspects of the surface EC' mechanism considered under voltammetric conditions can be found elsewhere.[3,13,15,17,19,21–23] We have recently published a theoretical paper, while referring to several new aspects of the surface EC' mechanism under conditions of square-wave voltammetry.[21] Moreover, in Ref. [21] we proposed a new square-wave voltammetric methodology to evaluate the kinetics of the surface electrode reaction under a constant scan rate. In this communication we describe a time-independent method for the determination of reaction rate of the chemical step of an EC' mechanism in protein-film cyclic staircase voltammetry. We used the cyclic voltammetry as a working technique, since it is a very popular electrochemical technique for mechanistic and thermodynamic studies of various electrode mechanisms.[1,4,16,17,23–26]

MATHEMATICAL MODEL

In the mathematical model, we consider a quasi-reversible surface electrode reaction of type Ox(ads) + ne^- ⇌ Red(ads), in which the product R undergoes a follow-up regenerative chemical reaction with dissolved non-electroactive species S. Both electroactive enzyme forms Ox and Red are strongly immobilized (adsorbed, ads) on a working electrode surface forming a monomolecular layer, and the mass transport occurring *via* diffusion is negligible. The chemical reaction between Red(ads) and S regenerates the starting material Ox(ads) on the electrode surface. Common schematic representation of the surface EC' mechanism is as follows:

Electrochemical (E) step:
   Ox(ads) + ne^- ⇌ Red(ads)

Catalytic (C') step:
   Red(ads) + S $\xrightarrow{k_c}$ Ox(ads) + side products
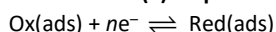
Initially, only the redox active adsorbates Ox are present in the electrochemical cell. By S we assign an electrochemically inactive compound (*i.e.* the Substrate) present in dissolved state in solution. The bulk concentration of S is assumed to be much higher than the initial concentration of all redox adsorbates Γ^*Ox). Consequently, the chemical step (C') in the considered redox mechanism is assumed to be of pseudo-first order. k_c (s^{-1}) is a pseudo first order catalytic rate constant. This parameter is related to the bulk concentration of substrate S as follows $k_c = k_{cat} \, c$(S). In the last equation, k_{cat} is the real chemical (*i.e.* catalytic or Michaelis–Menten) rate constant with units $mol^{-1} \, cm^3 \, s^{-1}$, while c(S) is the molar concentration of substrate S. We assumed that the electron transfer coefficient of cathodic and anodic step are equal and set to 0.5. The number of exchanged electrons is set to $n = 2$ in all simulations. All cyclic voltammograms are recorded by a scan toward the switching potential having more negative values. The theoretical calculations have been performed by using the MATHCAD 14 software. We give in the Appendix of this work a complete MATHCAD working sheet for the considered redox mechanism that can be freely used by everyone.

RESULTS AND DISCUSSION

We considered in this work the surface EC' mechanism under conditions of cyclic staircase voltammetry. More detailed information about the mathematical model and the algorithm applied can be found in Refs. [13,15,20,21] and in the Appendix at the end of this work. Calculated cyclic voltammograms are function of applied scan rate, the temperature T, the potential step of the staircase ramp dE, the number of electrons exchanged-n, and the electron transfer coefficient α tammograms are affected by two dimensionless parameters λ and γ. The kinetics of the electrode reaction Ox(ads) + ne^- ⇌ Red(ads) is portrayed via the dimensionless kinetic parameter-λ, defined as $\lambda = k_s \tau$. Here, k_s is the standard rate constant of electron transfer (s^{-1}), and τ is the duration of the potential step of the potential ramp. The rate of the chemical regenerative step (C') is represented via dimensionless catalytic parameter-γ ($\gamma = k_c \tau$), where k_c is the rate constant of the chemical regenerative reaction. The dimensionless current of calculated cyclic voltammograms Ψ is defined as: $\Psi = I \tau \, [n \, F \, A \, \Gamma^*(Ox)]^{-1}$. Here, I is the symbol for the current (Amperes), F is the Faraday constant (96485 C mol^{-1}), A is the working electrode surface area (cm^2), and Γ^*(Ox) is the initial surface concentration of Ox species. Note again that k_c is a pseudo-first order rate constant of the catalytic reaction (C') that is defined as $k_c = k_{cat} \, c$(S), where k_{cat} is the real rate constant of the chemical catalytic reaction ($mol^{-1} \, s^{-1}$ L), while c(S) (mol L^{-1}) is the molar concentration of catalyzing agent S present in dissolved state. The cathodic currents in the theoretical model are defined as "negative". In absence of catalyzing agent S, the inter-conversion between both adsorbed forms of electroactive enzyme Ox and Red is in

accordance with the applied potential. This gives rise to unperturbed cyclic voltammograms typical for a simple surface electrode reaction Ox(ads) + ne^- ⇌ Red(ads) (Figure 1A). When a catalyzing agent S is present in significant concentration in the system, then we observe quite different features in calculated voltammograms (curves 2–6, Figure 1B). As the catalyzing agent S reacts with the reduced form of enzyme Red, then initial enzyme form Ox gets chemically regenerated on the electrode surface. In the time-frame of current measurement in cyclic voltammetry, this additional material of the enzyme form Ox undergoes extra reduction at the working electrode. The voltammetric outcome of the catalytic regeneration of Ox is portrayed in elevating of the cathodic current branch, which is followed by changes in
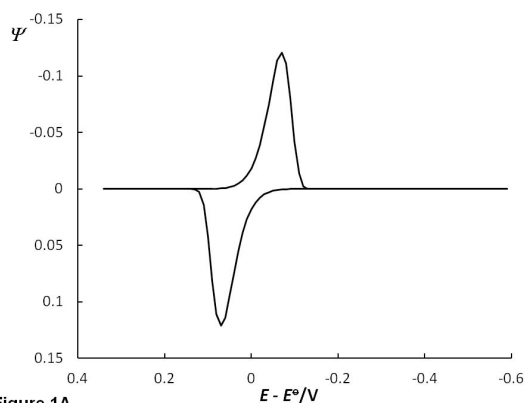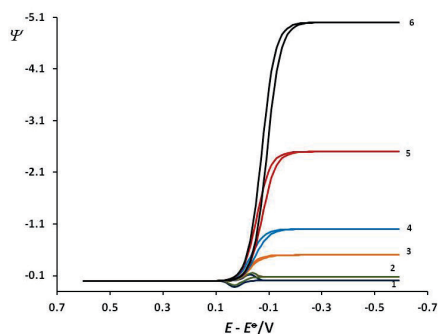

Figure 1A

Figure 1B

Figure 1. (A) Cyclic voltammogram of a surface EC' mechanism calculated in absence of substrate S. The simulation parameters are: temperature $T = 298$ K; dimensionless electrode kinetic parameter $\lambda = 0.2$; number of electrons $ne^- = 2$; electron transfer coefficient $\alpha = 0.5$, potential step d$E = 4$ mV, time increment $\tau = 0.05$ s. (B) Effect of dimensionless catalytic parameter γ on the features of calculated cyclic staircase voltammograms. Presented cyclic voltammograms are simulated for: $\gamma = 0.005$ (1); 0.075 (2) ;0.5 (3); 1 (4); 2.5 (5) and 5 (6). Other simulations parameters were identical as those for Figure 1A.

anodic currents, too (curves 2–5 in Figure 1B). This happens in potential regions in which the catalytic chemical reaction starts to exhibit its effect to electron transfer step of the electrode reaction (it starts at potentials of +50 mV of $E^o_{Ox/Red}$ and continues in further negative potentials). As the rate of the catalytic reaction increases, alongside the changes in cathodic branches of cyclic voltammograms, observable modifications occur in the anodic branches, too. When the rate of the regenerative catalytic reaction is very high, both currents branches of the cyclic voltammograms become "cathodic", with magnitudes very close to each other (curves 3 to 6 in Figure 1B). This event happens when the kinetics of chemical regenerative reaction S + Red(ads) → Ox(ads) becomes significant over the kinetics of the electrode reaction Red(ads) – ne^- → Ox(ads), roughly for $\gamma/\lambda > 2$. Under such circumstances, almost entire amount of reduced enzyme form Red, very quickly converts to the initial oxidized form of enzyme Ox. As a consequence, multiple occurrence of the electrode reaction Ox(ads) + ne^- → Red(ads) takes place at all applied potentials, resulting in "steady-state" cyclic voltammograms (curves 3 to 6, Figure 1B).[13,17,22] Recognizable principle for the steady-state voltammetry is the insensitivity of the "catalytic" cyclic voltammograms by decreasing of the scan rate.

In our recent work[21] we discovered several new voltammetric features of the EC' mechanism. We showed that access to the kinetics of electron transfer step of electrode reaction can be achieved by altering the rate of the chemical regenerative reaction in time-independent voltammetric experiments. In this work, we focus mainly on the features of the catalytic steady-state cyclic voltammograms as a function of the parameters affecting the electrode reaction of a surface EC' mechanism. Shown in Figure 2a–c is the effect of the electron transfer parameters of the electrode reaction on the simulated cyclic voltammograms. All the voltammograms are simulated for a constant scan rate and significant rate of the catalytic reaction ($\gamma = 0.5$) at room temperature ($T = 298$ K). Apparently, just some of the features of simulated cyclic voltammograms are affected by the parameters considered. Specifically, the kinetic parameter of the electrode reaction λ (Figure 2A), the electron transfer coefficient α (Figure 2B), as well as the number of electrons exchanged n (Figure 2C) affect the catalytic cyclic voltammograms of surface EC' mechanism in some parts only. Explanations about the effect of these parameters on the relevant features of catalytic cyclic voltammograms can be found elsewhere.[13,17,19–21,27] In spite of these effects, the maximal current magnitudes of catalytic cyclic voltammograms (see the encircled regions of the voltammograms in Figures 2A–C) do not depend on λ, α and n. The maximal ("plateau", or limiting) current magnitude of catalytic cyclic voltammograms depends solely on the rate of the chemical regenerative reaction.
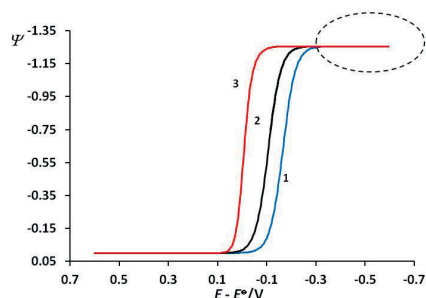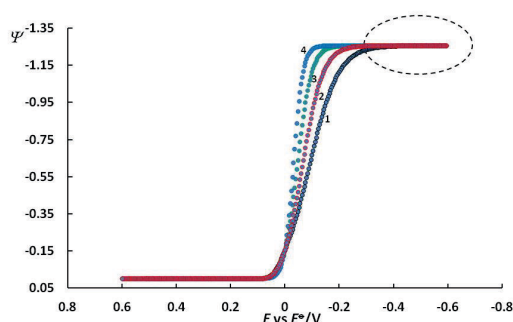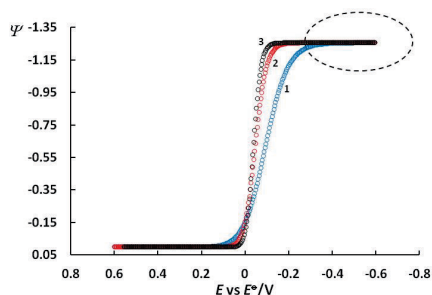
Figure 2A

Figure 2B

Figure 2C

Figure 2. (A) Effect of the kinetics of the electrode reaction (λ) on the features of cyclic voltammograms, calculated for λ= 0.002 (1); 0.02 (2) and 2 (3). (B) Effect of the electron transfer coefficient of electrode reaction α on the features of the cyclic voltammograms. Voltammograms are calculated for α = 0.25 (1); 0.5 (2) and 0.75 (3). C. Effect of the number of exchanged electrons on the features of the cyclic voltammograms. Presented voltammograms are calculated for ne^- = 1 (1); 2 (2) and 3 (3). Other simulation parameters were: γ = 1.256, λ= 0.2 (for B and C), *T* = 298 K; ne^- = 2 (for A and C) and α = 0.5 (for A and B), d*E* = 10 mV, τ = 0.005 s.
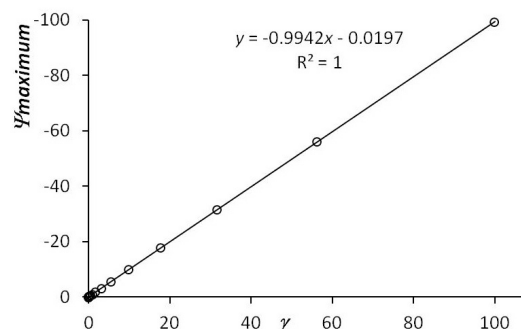

Figure 3. Dependence of maximal catalytic currents of simulated cyclic steady-state voltammograms as a function of dimensionless catalytic parameter γ. All the other simulation parameters were the same as those in Figure 1.

The region of very negative potentials (approximately about −200 mV and more negative than the value of the standard redox potential $E^o{}_{Ox/Red}$) is a section in which the kinetics of the electrode reaction Ox(ads) + ne^- → Red(ads) is very high. Accordingly, in that potential region, the entire oxidized form of the enzyme Ox on the electrode surface will be very quickly electrochemically converted to the reduced enzymatic form Red at the working electrode. Consequently, the current magnitude of the steady-state cyclic catalytic voltammograms in this sector of applied potentials depends exclusively on the rate by which the catalytic step regenerates chemically the Ox form of the enzyme. For that reason, these fragments of the steady state cyclic voltammograms (encircled regions in Figure 2A-C) can be explored for developing an approach to evaluate kinetic parameters related to the catalytic reaction of a surface EC′ mechanism. Shown in Figure 3 is the dependence between the maximal current magnitudes of simulated catalytic cyclic voltammograms Ψmax and the catalytic parameter γ. The slope of the linear dependence between the Ψmax and γ (R^2 = 1) allows direct and simple estimation of the real value of the catalytic rate constant (k_{cat}). As it is well known, the rate of the chemical catalytic reaction (under constant scan rate and constant temperature) can be affected via the substrate concentration S (the catalyzing agent). This feature can be explored to determine the real catalytic rate constant of a given enzyme-substrate system, by evaluating the maximal current magnitudes of the steady-state cyclic voltammograms recorded for several concentrations of the catalyzing agent S.

CONCLUSION

Cyclic voltammetry applied to a protein-film set-up is

important approach because it produces a comprehensible visual picture for the electrochemical activity of a given enzyme as a function of the applied potential. Among the mechanisms studied with protein-film cyclic voltammetry, the surface EC' mechanism is one of the most relevant electrode systems that is linked to the enzyme-substrate interactions.[3–7,19] Various aspects of the surface EC' mechanism have been published so far, and relevant time-dependent voltammetric methods have been developed for the kinetics and thermodynamic evaluations applicable to important enzymatic systems.[3–8,13,15,17,19,21] Recently, couple of scientific contributions referred to new voltammetric methods for evaluating the rate constants of electrode reactions from time-independent experiments.[21,28–30] In this work, we present a simple and time-independent cyclo-voltammetric approach for evaluating the kinetic rate constant of catalytic reaction of a EC' mechanism featuring quasi-reversible electrode reaction in protein-film set-up. At large overpotentials, the limiting current of catalytic cyclic steady-state voltammograms is independent of applied potential, but it depends wholly on the rate of chemical regenerative reaction (Figures 2 and 3). Moreover, we show that the limiting catalytic current is linear function of the dimensionless catalytic parameter γ (see Figure 3). If we recall that the dimensionless catalytic parameter γ is defined as $\gamma = k_c \tau$, and $k_c = k_{cat}c(S)$, then we can set a simple experimental manner to reconstruct the theoretical dependence from Figure 3. This can be done by designing a protein-film voltammetric experiment in which we adjust the concentration of the catalyzing agent $c(S)$ only. When an experimental situation of steady-state voltammograms is reached, then we measure the magnitude of the limiting (maximal) catalytic current I_{max} and we plot I_{max} as a function of the molar concentration of the catalyzing agent-$c(S)$. The slope of the linear dependence I_{max} *vs.* $c(S)$ is defined as: *slope* = $|(k_{cat})[nFA\Gamma^*(Ox)\tau^{-1}]|$. If number of electrons exchanged n and the surface concentration $\Gamma^*(Ox)$ are known, and if we work under constant scan rate (then we will know the value of τ), then we get the value of k_{cat} in a very simple procedure from the slope of the I_{max} *vs.* $c(S)$ dependence. Proposed methodology allows simple estimation of k_{cat}, and for that we do not need to know the magnitudes of the parameters related to the electron transfer step of enzyme's electrode reaction (k_s and α). The determination of the catalytic rate constant k_{cat} is very important for all enzymatic systems, since it reflects the magnitude of the Michaelis-Menten constant. For the determination of surface concentration $\Gamma^*(Ox)$, one can use some of the methods described in [23, 31, 32]. In our last work[33] we showed that for dissolved redox proteins, I_{max} is a linear function of the square-root of $c(S)$. By the EC' mechanism of dissolved redox proteins,[33] the mass transfer of both redox enzyme forms is achieved

via diffusion, and this phenomenon contributes to the features of limiting currents. For the surface EC' mechanism, the diffusion does contribute to the limiting current features of cyclic voltammograms since redox adsorbates stay firmly immobilized on working electrode surface at all potentials applied. Therefore, both approaches (*i.e.* the protein-film voltammetry and the voltammetry of dissolved redox proteins) have distinguishable arguments for the determination of the Michaelis-Menten constant.

Supplementary Information. Supporting information to the paper is attached to the electronic version of the article at: http://doi.org/10.5562/cca3383.

REFERENCES

[1] A. J. Bard, L. R. Faulkner, *Electrochemical Methods Fundamentals and Applications*, 3rd edition, John Wiley & Sons, Inc. **2004**.

[2] J. M. Savéant, *Elements of Molecular and Biomolecular Electrochemistry: An Electrochemical Approach to Electron Transfer Chemistry*, Hoboken, NJ, Wiley, **2006**.

[3] J. N. Butt, F. A. Armstrong, *Bioinorganic Electrochemistry*, (Eds. O. Hammerich, J. Ulstrup), Springer, Netherlands, **2008**.

[4] F. A. Armstrong, H. A. Heering, J. Hirst, *Chem. Soc. Rev.* **1997**, *26*, 169.

[5] C. Léger, P. Bertrand, *Chem. Rev.* **2008**, *108*, 2379.

[6] F. A. Armstrong, *Encyclopedia of Electrochemistry*, vol. 9 (Eds. A. J. Bard, M. Stratmann, G. S. Wilson), Wiley VCH, Weinheim, **2002**.

[7] F. A. Armstrong, *Bioelectrochemistry: Principles and Practice*, vol. 5, (Eds. G. Lenaz, G. Milazz), Birkhauser Verlag AG, Basel, **1997**.

[8] R. Gulaboski, M. Lovrić, V. Mirčeski, I. Bogeski, M. Hoth, *Biophys. Chem*. **2008**, *137*, 49.

[9] J. Hirst, *Biochem. Biophys. Acta* **2006**, *1757*, 225.

[10] F. A. Armstrong, N. A. Belsey, J. A. Cracknell, G. Goldet, A. Parkin, E. Reisner, K. A. Vincent, A. F. Wait, *Chem. Soc. Rev.* **2009**, *38*, 36.

[11] R. Gulaboski, *J. Solid State Electrochem.* **2009**, *13,* 1015.

[12] R. Gulaboski, V. Mirceski, I. Bogeski, M. Hoth, *J. Solid State Electrochem.* **2012**, *16*, 2315.

[13] V. Mirceski, R. Gulaboski, *J. Solid State Electrochem*. **2003**, *7,* 157.

[14] R. Gulaboski, P. Kokoskarova, S. Mitrev, *Electrochim. Acta* **2012**, *69*, 86.

[15] P. N. Barlett, *Bioelectrochemistry-Fundamentals, Experimental Techniques and Applicatio*, Wiley, Chichester, **2008**.

[16] R. G. Compton, C. E. Banks, *Understanding Voltammetry*, Willey, **2007**, pp. 233–284,

[17] R. Gulaboski, L. Mihajlov, *Biophys. Chem*. **2011**, *159*, 1.

[18] V. Mirceski, S. Komorsky-Lovrić, M. Lovrić, *Square-Wave Voltammetry: Theory and Application*, (Ed. F. Scholz) 2nd Ed., Berlin, Springer, **2007**.

[19] V. Mirceski, R. Gulaboski, M. Lovric, I. Bogeski, R. Kappl. M. Hoth, *Electroanal.* **2013**, *25*, 2411.

[20] T. M. Saccucci, J. F. Rusling, *J. Phys. Chem. B* **2001**, *105*, 6142.

[21] R. Gulaboski, V. Mirceski, *Electrochim. Acta* **2015**, *167*, 219.

[22] S. E. Creager, T. T. Wooster, *Anal. Chem*. **1998**, *70*, 4257.

[23] J. Wang, *Analytical Electrochemistry,* 2nd Ed., Wiley VCH, **2000**.

[24] V. D. Parker, *Linear sweep and cyclic voltammetry, Comprehensive Chemical Kinetics*, **1986**, *26*, 145.

[25] L. K. Bieniasz, J. Gonzalez, A. Molina, E. Laborda, *Electrochim. Acta* **2010**, *56*, 543.

[26] S. Komorsky-Lovrić, M. Lovrić, *Anal. Chim. Acta* **1995**, *305*, 248.

[27] D. Jadresko, D. Guzijewski, V. Mirceski, *Chem. Electrochem.* **2018**, *5*, 187.

[28] V. Mirceski, D. Guzijewski, M. Bozem, I. Bogeski, *Electrochim. Acta* **2016**, *213*, 520.

[29] V. Mirceski, E. Laborda, D. Gruzijewski, R. G. Compton, *Anal. Chem*. **2013**, *85*, 5586.

[30] R. I. Masel, *Principles of Adsorption and Reaction on Solid Surfaces*, Wiley, New York, **1996**.

[31] K. Christmann, *Introduction to Surface Physical Chemistry*, Steinkopff-Verlag, Darmstadt, Germany, **1991**.

[32] G. Ertl, J. Küppers, *Low-energy electrons and surface chemistry*, 2. Auflage, Verlag Chemie, Weinheim, **1985**.

[33] R. Gulaboski, S. Petkovska, *Anal. Bioanal. Electrochem*. **2018**, *10*, 566.