Electrogravimetric Real-Time and in Situ Michaelis−Menten Enzimatic Kinetics: Progress Curve of Acetylcholinesterase Hydrolysis

Paulo R. Bueno,*,† Ailton M. Watanabe,† Ronaldo C. Faria,‡ Márcio L. Santos,† and Carla S. Riccardi†

Instituto de Química, Universidade Estadual Paulista, CP 355, 14800-900, Araraquara, São Paulo, Brasil and Departamento de Química, Universidade Federal de São Carlos, CP 676, 13560-905, São Carlos, São Paulo, Brasil

Received: July 7, 2010; Revised Manuscript Received: October 13, 2010

A piezoelectric detection of enzyme-modified surface was performed under Michaelis−Menten presumptions of steady-state condition. The approach herein presented showed promise in the study of enzymatic kinetics by measuring the frequency changes associated with mass changes at the piezoelectric crystal surface. Likewise, real-time frequency shifts, that is, $d\Delta f/dt$, indicated the rate of products formation from enzymatic reaction. In this paper, acetylcholinesterase was used as the enzymatic model and acetylcholine as substrate. The enzymatic rate has its maximum value for a short time during the kinetic reaction, for instance, during the first ten minutes of the reaction time scale. The values found for the kinetic constant rate and Michaelis−Menten constant were $(1.4 \pm 0.8)\ 10^5\ s^{-1}$ and $(5.2 \pm 3)\ 10^{-4}$ M, respectively, in agreement with the values found in classical Michaelis−Menten kinetic experiments.

Introduction

During the last ten years, the biomolecules immobilization at solid−liquid interfaces has been very important in medical diagnosis applications.[1−5] For instance, the approach for antigen−antibody interactions is already well reported.[1−10] The experimental framework involving measuring changes in adsorbed masses and viscoelastic properties (of the adsorbed material) via differences in the frequency and decay oscillation of a piezoelectric quartz crystal is, for instance, useful in measuring DNA absorption, hybridization, and cell adhesion as well.[9,11,12]

On the one hand, the monitoring of an enzymatic reaction has not been fully exploited up to now in terms of real-time mass change at solid−liquid interfaces. The literature has reported indirect assays to monitor enzymatic reactions as a function of substrate concentrations.[13−18] On the other hand, the enzymatic kinetic models in biochemistry have been provided by Michaelis−Menten formalism.[18,19] The Michaelis−Menten kinetic model refers mainly to a set of key assumptions shared with the original development of the theory and provides the framework for treating various allosteric mechanisms, which generate behavior that is often referred to as "no-hyperbolic", receptor-binding and transport mechanisms, and growth of microorganisms.[18−20] The Michaelis−Menten formalism assumes that the elementary steps of an enzymatic mechanism follow those of traditional mass-action kinetics.[19]

The global mechanism is considered to operate far from thermodynamic equilibrium, in which a single molecule of free substrate (*S*) associates to form a specific enzyme−substrate complex (*ES*) that dissociates into free enzyme (*E*) and either free product (*P*)[19]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_c} E + P \tag{1}$$

The monomolecular rate constants are given by k_c and k_{-1}, and bimolecular rate constant is given by k_1.

The kinetic equations representing this mechanism are a composite description arrived at by treating the elementary steps according to traditional chemical reactions[19]

$$\frac{d[ES]}{dt} = k_1[E][S] - [ES](k_{-1} + k_c) \tag{2}$$

$$\frac{d[P]}{dt} = v_p = k_c[ES] \tag{3}$$

These equations are nonlinear, and the inability to solve them readily at the turn of the century led to the quasi-steady-state assumption, whereby one considers the dynamics of the enzyme−substrate complex to be so fast that its concentration can be treated as if it were in steady-state regime (or in equilibrium).[19] This is equivalent to setting the time derivative of the enzyme−substrate concentration to zero (or $[ES]/[E] = [S]/K_m$) and reducing the differential equation to an algebraic one (steady-state assumption). K_m is the Michaelis−Menten constant, which is considered mathematically equivalent to equilibrium dissociation constant, that is K_s, for [*ES*] complex (K_s) especially when $k_{-1} \gg k_c$.[19] The algebraic equation is then combined with the constraint equation for total enzyme amount, which states that the concentration of total enzyme concentration must be equal to the concentration of enzyme−substrate complex.[19] The result is a set of two algebraic equations that are linear in the two variables [*E*] and [*ES*]

$$0 = k_1[E][S] - [ES](k_{-1} + k_c) \tag{4}$$

* To whom correspondence should be addressed. Phone: +55 16 33016642. Fax: +55 16 33016692. E-mail: prbueno@iq.unesp.br.
† Universidade Estadual Paulista.
‡ Universidade Federal de São Carlos.

10.1021/jp106274m
Published on Web 11/17/2010

$$[E]_t = [E] + [ES] \quad (5)$$

This set of linear equations is readily solved to yield the concentration of the enzyme−substrate complex as a function of the rate constant for the elementary steps and of the concentration of free substrate and total enzyme. The resulting expression (enzymatic reaction velocity, v_p) is conventionally represented as a nonlinear (rational) function of substrate concentration and two aggregate parameters, the maximum rate (r_m) and Michaelis constant (K_m)[19]

$$v_p = \frac{r_m[S]}{[S] + K_m} \quad (6)$$

The kinetic parameters of the above relationship can be experimentally accessed by constructing an analytical curve by varying the substrate concentration from saturated to nonsaturated concentration and different forms of plot representation of eq 6 are possible and useful for different purposes.[19]

The goal of the present work is to show that modern electrical probing techniques can be useful and easily access the parameters of eq 6 accurately. Therefore, as in the case of protein adsorption studies at the piezoelectric quartz crystal surface, it is very desirable to develop experimental methodologies capable of monitoring the enzymatic process in real-time. This could open a remarkable avenue to the study of enzymatic kinetics, since almost all processes in a biological cell need enzymes to occur at significant rates and especially because the majority of the processes occur at a heterogeneous boundary steady of homogeneous conditions, as is the case of classical Michaelis−Menten enzyme kinetics.

Experimental Section

Apparatus. QCM-D (Quartz Crystal Microbalance with Dissipation Monitoring) experiments in Q-SENSE E4 equipment were carried out using 4.95 MHz AT-cut quartz crystals (Q-SENSE QSX 301) with gold electrodes on both sides. Prior to the experiments, the electrode surface was cleaned in piranha solution 4:1 H_2SO_4/H_2O_2 (30% v/v). After rinsing, the crystal was placed in a flow cell with one face in contact with the air and the other with the solution. A peristaltic bomb (ISMATEC IPC 4 channels) was used to control the flow in all of the experiments.

Preparation of Acetilcolinesterase-Functionalized Electrode. The methodology employed for enzyme immobilization was based on monolayer assembly on the gold surface of a piezoelectric quartz crystal transducer.[21] The piezoelectric quartz crystal was incubated for 3 h in a solution containing 1.0×10^{-3} mol L^{-1} 11-mercaptoundecanoic acid (MUA, Sigma Co.) and 100×10^{-3} mol L^{-1} of 2-mercaptoetanol (ME, Sigma Co.) in ethanol as solvent. Following, the crystal was washed with absolute ethanol and 0.1 M phosphate buffer saline (PBS) prepared in NaCl 0.15 M, pH 7.4 and dried with nitrogen. Previously to immobilization process, the quartz crystal was left resting for about 18 h. After this, the quartz crystal was incubated for 2 h in EDC (Fluka)/NHS (Fluka) solution 1.0×10^{-2} and 2.0×10^{-2} mol L^{-1}, respectively. After that, it was washed with PBS, aiming to remove possible residual molecules.

Finally, the quartz crystal was exposed to the enzyme solution (50 units mL^{-1}, Sigma Co.) for 2 h and then rinsed with PBS. All functionalized steps were performed under static mode, that is, flow-off mode.

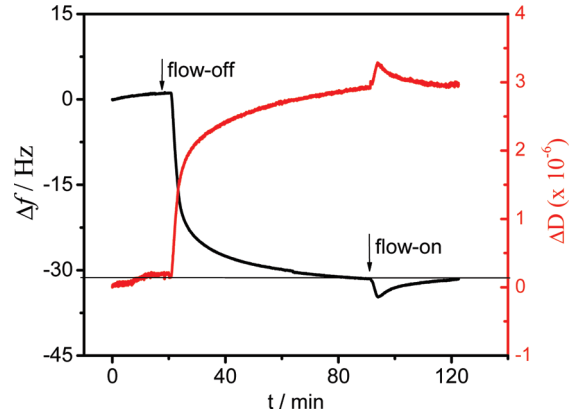

Figure 1. Frequency shift (Δf) and dissipation shift (ΔD) as a function of time during AChE immobilization process. The enzyme was injected in the time indicated with an arrow where the flow was turned off. The flow with pure buffer solution was turned on about 90 min as also indicated in the figure.

In fact, it can be seen from the real-time analysis of enzyme immobilization process shown in Figure 1 that all signal frequency variation, monitored during immobilization process, indicates that the attached enzymatic mass binds specifically since no frequency variation was detected after washing process (there is not nonspecific binding). Also, from Figure 1 it was detected a frequency variation of about −30 Hz for enzyme immobilization process. From this value and using eq 10, the concentration of acetylcholinesterase (AChE) immobilized in the self-assembled monolayer (SAM) of alkanethiols over the quartz crystal surface was inferred to be 540 ng cm^{-2}. Provided that the active electrode area is 0.785 cm^2, it was obtained a total enzyme mass of about 424 ng. As the cell volume is 40 μL, the total enzymatic volumetric concentration was about 0.01 g L^{-1}, that is, the estimated enzymatic activity of the electrode surface is 2.7 units cm^{-2}.

Enzymatic Activity Measurements. The assembled AChE-QCM piezoelectric sensor was immersed in 50 mL of 0.1 M phosphate buffer pH 7.4 and placed in the thermostatted cell at (37.0 ± 0.5) °C. The flow rate was adjusted to 60 μL min^{-1}. For all experiments, 50 mM potassium phosphate buffer pH 7.4 was used. Before measurements, the system was submitted to flow for 20 min to stabilize the baseline to ±1 Hz. Finally, the quartz crystal was exposed to the saturated substrate (acetylcholine chlorine, ACh) solution concentration of 0.5 M (500 times higher than enzymatic mass concentration). The measurements were made in quadruplicates to verify reproducibility. Each measuring cycle consisted of 5 min sample injection, 60 min interaction step (flow-off) and 20 min washing step (buffer flow). Frequency changes (Δf) as a function of time were obtained at a step time of 3 s. Time evolutions were obtained by plotting Δf values (hertz) versus time (minutes).

The monolayer enzyme activity was confirmed by studying the behavior of the electrode using BSA protein as negative control and by this we have assumed that immobilized enzyme reacts only with the substrate and no other effect was related to the frequency variation during the experiments. Therefore, BSA was added in the AChE-modified quartz crystal in the place of the substrate molecule. The electrode response was showed in Figure 2. As can be seen, there is no frequency variation (or mass variation) as function of time during 80 min, assuring that when ACh is added, the frequency variation is only due to enzyme activity as predicted by eqs 10 and 11 so that the enzyme kinetic can be studied (see the responses of Figures 2 and 5, this last further discussion herein).

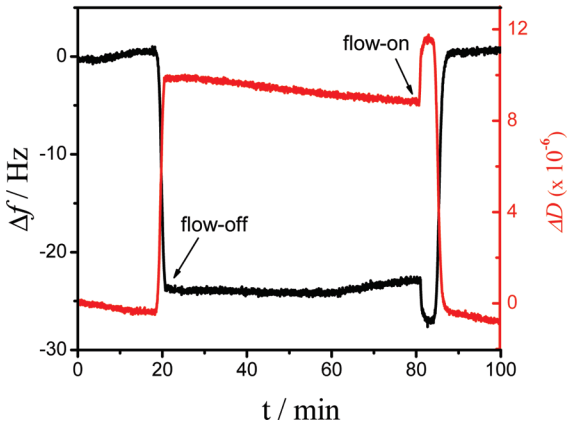

Figure 2. Frequency shift (Δf) and dissipation shift (ΔD) as a function of time during immobilization process of the BSA over the QCM-chip enzimatically functionalized. The enzyme was injected in the time indicated with an arrow where the flow was then turned off. The flow with pure buffer solution was turned on about 80 min as also indicated in the figure. There were no frequency variations that can be translated into enzymatic activity as expected (i.e., the response is completely flat during the process).

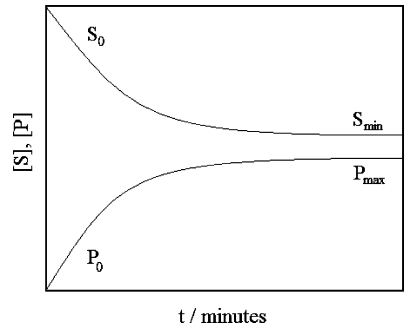

Figure 3. Changes in S and P concentrations as a function of time from initial values ([S_0] and [P_0]) to final values ([P_{max}] and [S_{min}]).

Results and Discussion

Electrogravimetric Enzymatic Kinetic Theory. One of the limitations of the Michaelis−Menten equation (eq 6) is that the consumption of S or formation of P is not possible to be detected directly. It means that a real-time progress curve is impossible to be obtained by traditional kinetic methodologies.[19] For the conversion of S to products P, the general shape of the progress curve is that of first-order exponential decrease in substrate concentration, see Figure 3, that is

$$[S - S_{min}] = [S_0 - S_{min}]e^{-t/\tau} \quad (7)$$

or that of the first-order exponential increase in products concentration

$$[P - P_0] = [P_{max} - P_0](1 - e^{-t/\tau}) \quad (8)$$

where [S_0], [S_{min}], and [S] correspond, respectively, to initial substrate concentration ($t \rightarrow 0$), minimum substrate concentration ($t \rightarrow \infty$), and substrate concentration at time t. On the other hand, [P_0], [P_{max}], and [P] correspond, respectively, to initial products concentration ($t \rightarrow 0$), maximum product concentration ($t \rightarrow \infty$), and products concentration at time t, see Figure 3. τ is the relaxation time (inverse of relaxation frequency) of the kinetic process.

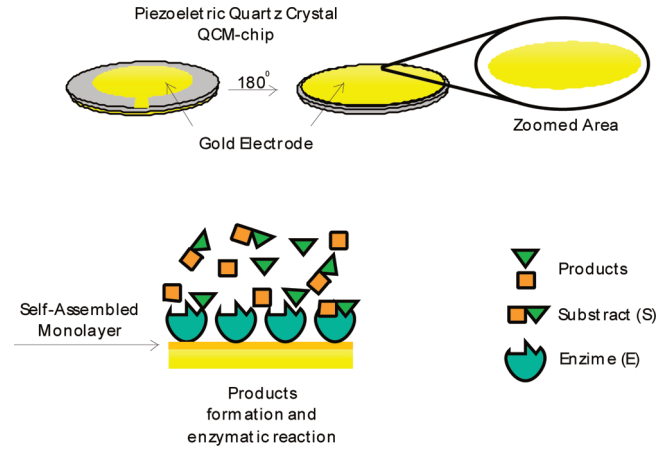

Figure 4. Schematic representation of the electrogravimetric approach measurement of Michaelis−Menten enzymatic kinetics. At steady-state regime, the piezoelectric quartz crystal response over time is directly proportional to the products rate of the enzymatic reaction. For short times, the rate reaction is constant according to the kinetic model. The experimental example used to illustrate this approach was the based on the acetylcholinesterase rate conversion of acetylcholine into acetyl plus choline, which is described hereby.

The enzymatic rate of the reaction, or reaction velocity, corresponds to the instantaneous slope of the progress curve

$$v_p = -\frac{dS}{dt} = \frac{dP}{dt} \quad (9)$$

On the basis of the last assumption, in the present work we propose a methodology to monitor enzymatic reactions in real-time by using electrogravimetric technique (more commonly referred to as QCM), that is, a piezoelectric AT-cut quartz crystal enzymatic functionalized electrode capable to monitor the frequency changes of a piezoelectric transducer in real-time (see Figure 4 for illustrative purpose).

If a rigid layer is evenly deposited on metal-electrode of quartz crystal, the resonant frequency will decrease, that is $-\Delta f$, proportionally to the mass (Δm) of the adsorbed layer. The relationship between Δm and Δf for ideal former situation was derived by Sauerbrey[22] and is described by

$$\Delta f = -n\frac{2f_0}{A\sqrt{\mu_q \rho_q}}\Delta m = -nC_f\Delta m \quad (10)$$

in which f_0 is the fundamental frequency of the quartz resonator, A is the piezoelectric active area, n is the overtone number of resonant frequency (i.e., $n = 1$ for the fundamental frequency) and ρ_q and μ_q are the density and shear modulus of the AT-cut crystal.[22] Nanograms variation is possible to be measured by using QCM technique.[4] Therefore, based on the QCM technique fundaments we have developed a useful approach to study enzymatic kinetics according to the set of key assumption provided by the original Michaelis−Menten formalism. The dissipative factor of the quartz crystal can be measured simultaneously to the frequency variation and problems related to the viscosity of the solution on the mass variation.[4] However, this will be a detail discussion in a further work concerning enzymatic reactions aspect.

Herein we report that the proposed methodology is very useful and simple, consisting on real-time monitoring of enzymatic activity by means of piezoelectric detection. The positive

variation of quartz crystal frequency (mass losses) as function of time, after steady-state condition is reached, is proportional to the monomolecular enzymatic reaction rate, that is, $v_p = dP/dt = k_c[ES]_s$ in which $[ES]_s$ is the concentration of enzyme−substrate on the quartz crystal surface as a time function. The rate in which products is formed is proportional to the rate of frequency variation, that is, the mass losses of the quartz crystal after the steady-state condition is reached for $[S]$ in excess or $[S] \gg K_m$ in eq 6. Therefore, if $[S]$ is in excess with respect to $[E]_s$ (the total concentration of enzyme immobilized in the quartz crystal surface) the steady-state condition is reached quickly compared to reaction time scale. During reaction progress and steady-state (or affinity equilibrium between enzyme and substrate), the unique frequency variation expected is that related to

$$ES_s \xrightarrow{k_c} E_s + P \tag{11}$$

and in this situation the pattern predicted by eq 8 can be directly measured in real-time provided that

$$v_p = \frac{dP}{dt} = (-nC_f)^{-1}\frac{d\Delta f}{dt} \tag{12}$$

indicating that the rate of products formation causes a mass decrease in the surface of the quartz crystal (i.e., the mass decreases and the oscillating frequency of the system increases). Considering eq 10, it can be stated that

$$\frac{d\Delta f}{dt} = -nC_f\frac{d\Delta m}{dt} = nC_f\frac{dP}{dt} = nC_f v_p \tag{13}$$

The derivative form of eq 8 leads to

$$\frac{dP}{dt} = \Delta P\tau^{-1}e^{-t/\tau} \tag{14}$$

where $\Delta P = P_{max} - P_0$ and for $t \rightarrow 0$ and considering that $P + S = 1$ (or $\Delta P = -\Delta S$). In terms of frequency variation, eq 14 turns into

$$\frac{d\Delta f}{dt} = (nC_f)^{-1}\Delta P\tau^{-1}e^{-t/\tau} \tag{15}$$

The equilibrium affinity constant between E and S is considered faster than the enzyme-complex consumption up to this point. The inhibition of the reaction by products and enzymatic instability were disregarded.

Note that, as expected, when $\tau \gg t$ or $t \rightarrow 0$ eq 14 tends to

$$v_p = \frac{dP}{dt} = \Delta P\tau^{-1} \tag{16}$$

or

$$v_p = \frac{d\Delta f}{dt} = (nC_f)^{-1}\Delta P\tau^{-1} \tag{17}$$

that is, the enzymatic rate is constant and maximum, meaning that $[P]$ is about zero; thus the reverse reaction can be considered to be negligible, and any possible inhibitory effects of products is not significant as well as any decrease on the saturation of enzyme.

The above assumption was tested by choosing the well-known AChE as our enzymatic model to be monitored. AChE was chosen not only because it is well-known enzyme, but because it is also related to Alzheimer disease whose treatment consist in the inhibition of this enzyme.[23−28] As a substrate it was elected the ACh, a low molecular mass substrate.

Experimental Electrogravimetric Progress Curve. First of all, it is important to observe that the self-assembled monolayer was constructed, as described in experimental section, consisting of two alkanethiols, that is, MUA and 2-mercaptoethanol in a proportion of 1:100, respectively. The MUA was used for AChE immobilization on the metal-electrode quartz crystal surface by means of the amine group present in the AChE structure with the carboxyl group present in the MUA. Thus, the 2-mercaptoetanol served as a spacing layer, that is, to space the immobilized enzyme molecules.[21]

Because of the proportion of 1:100, it can be seen that the transducer's functional surface provided a suitable environment to observe ACh−AChE interacton on the piezoelectric transducer surface. It also allows a sufficient distance between similar units of immobilized AChE on the sensing surface to prevent cross interaction of AChE molecules.[21] Furthermore, it is known that the AChE−ACh reaction is irreversible and kinetically rapid, allowing mass transport to be neglected after steady-state is reached.

The enzyme-modified quartz crystal was placed in the Q-SENSE cell under a continuous PBS flow until the frequency signal stabilized as described in experimental section and the total response over time is shown in Figure 5, represented by three different regions in time. The real-time binding study involved adding saturated amount of ACh enzymatic substrate to the bulk solution in contact with AChE-modified electrode surface. Figure 5a shows the frequency and dissipation shifts as a function of time for initial velocity of the enzymatic reaction. This region, stated as I, shows that when the PBS flowing over the enzyme functionalized piezoelectric surface was changed to a flow of ACh, a large decrease in frequency of −22 Hz was observed. This was likely to be caused by first, an initial rapid "bulk shift" due to the change in bulk solution (buffered to enzymatic substrate solution) and second because of enzymatic reaction started and steady-state was not yet reached. Figure 5a also shows the corresponding changes in dissipation (D), which increased about to 9×10^{-6} over around 20 min.

Note that the dissipation pattern as a function of time is similar but not equivalent to the pattern found for frequency change. Step I then corresponds to acetylcholine adsorption (enzyme substrate complex, S), whereas steps II and III indicate the acetylcholine hydrolysis (steady-state with flow-off) along the time course of the reaction. In the region corresponding to step II it is observed the reaction up to 100 min later after the startup and in region III is the reaction stabilization for time large than 700 min, that is for $t \rightarrow \infty$. As expected, the initial velocity is constant as in agreement to eq 14. During the early stages of an enzyme-catalyzed reaction, according to Michaelis−Menten enzymatic kinetics, the conversion of substrate to product is small and can be also considered to remain constant and effectively equal to initial substrate concentration ($[S_t] \sim [S_0]$) in agreement with the pattern described in Figure 1.

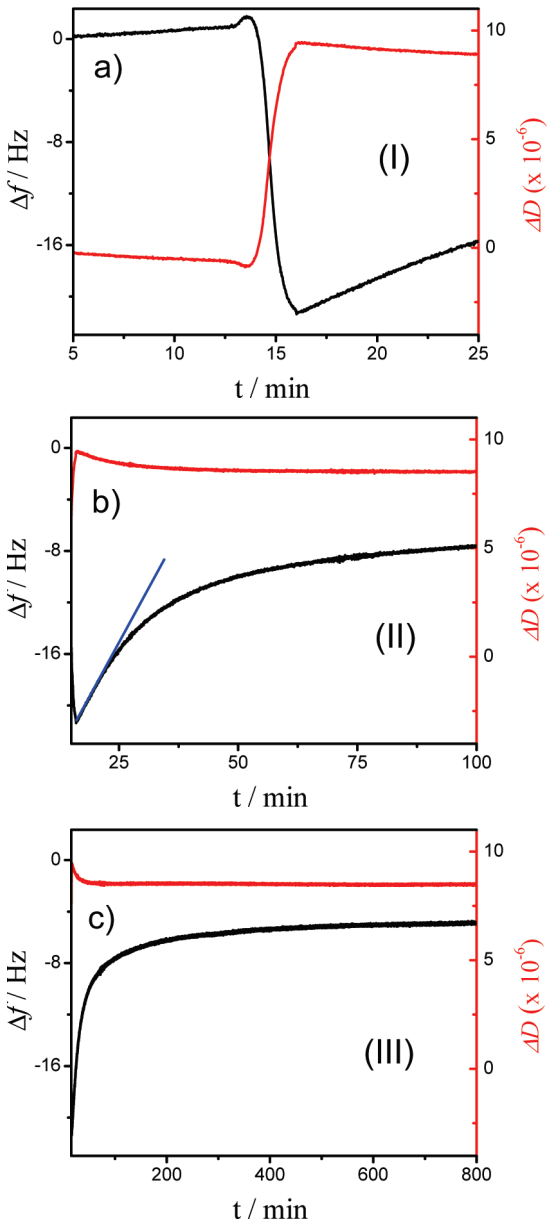

Figure 5. Steps I−III emphasize different processes during kinetic reactions. Step I is related to enzyme adsorption, that is, a step before the beginning of the enzyme reaction. Steps II and III, for instance, show the enzyme hydrolysis pattern obtained during the experimental time scale. Frequency (Δf) and dissipation (ΔD) changes measured by piezoelectric detection for AChE real-time enzymatic reaction. (a) ACh substrate injection and steady-state assumption. The region I correspond to initial velocity of enzymatic reaction and the condition of enzyme saturation by substrate. (b) Details of region II where the enzymatic reaction progress after enzyme is not any more saturated by the substrate (around 25 or 10 min of reaction), that is, the nonlinear region. In the nonlinear region, the enzymatic reaction starts to be dependent on the [*ES*]. (c) The entire reaction time and [P_{max}] around a frequency plateau of 6 Hz.

The initial velocity was calculated as $(1.4 \pm 0.8)\ 10^5\ s^{-1}$ for 37 °C, which is the value around that found for the hydrolysis of ACh by the AChE.[19] The value of τ calculated from eq 8 was estimated to be $4.2 \times 10^{-4}\ s^{-1}$. The values and errors were estimated from four reproducible experiments.

As previously discussed, Figure 5a shows the injection of [*S*] in 0.5 M to QCM-chip microreactor with an amount of enzyme previously immobilized on it and the steady state was reached, identified as a discontinuity in the derivative of $d\Delta f/dt$ at about 16 s. About this time, the $[ES]_s/[E]_s$ ratio ($\sim 1.1 \times 10^3$) was estimated by means of functionalized QCM-chip analysis considering a blank reference of [*S*] injection into a microreactor containing only the SAM to normalize the experiment, meaning that with this procedure the injection time and viscosity related problems were corrected.[2,29] The positive variation of quartz crystal frequency (mass losses) as function of time in the steady-state regime is proportional to the monomolecular maximum enzymatic reaction rate as demonstrated previously in eq 10. Therefore, if [*S*] is in excess with respect to $[E]_s$, the steady-state situation is reached quickly and can be monitored in real-time according to Michaelis−Menten presumption. From the values of $[ES]_s/[E]_s$ ratio experimentally calculated and with an excess concentration of 0.5 M for [*S*], the K_s was then easily estimated as $(5.2 \pm 3.0)\ 10^{-4}$ M.

Conclusions and Final Remarks

In conclusion, a quartz-crystal analysis of enzymatic functionalized QCM-chip was successfully proposed. Under steady-state condition, the frequency of piezoelectric quartz crystal shifts with time, that is, $d\Delta f/dt$, proportionally to the rate of products formation so that mathematically related to the enzymatic reaction rate from which the main enzymatic kinetics constants were calculated.

The hydrolysis electrogravimetric progress curve for acetylcholinesterase using acetylcholine as substrate was obtained in situ and in real-time. The proposed methodology will open an avenue to study enzymatic reactions by means of enzyme functionalized QCM-chip analysis.

Acknowledgment. This work was supported by the São Paulo state research funding agency (FAPESP). The authors also acknowledge the National Research Council (CNPq). We also thank the BIOTA program (FAPESP research center) and their researchers for useful discussion.

References and Notes

(1) Hook, F.; Ray, A.; Norden, B.; Kasemo, B. *Langmuir* **2001**, *17*, 8305.
(2) Hook, F.; Rodahl, M.; Brzezinski, P.; Kasemo, B. *Langmuir* **1998**, *14*, 729.
(3) Pohanka, M.; Kuca, K.; Kassa, J. *Neuroendocrinol. Lett.* **2008**, *29*, 755.
(4) Rodahl, M.; Hook, F.; Kasemo, B. *Anal. Chem.* **1996**, *68*, 2219.
(5) Ward, M. D.; Buttry, D. A. *Science* **1990**, *249*, 1000.
(6) Glasmastar, K.; Larsson, C.; Hook, F.; Kasemo, B. *J. Colloid Interface Sci.* **2002**, *246*, 40.
(7) Jimenez, C.; Leon, D. E. *Vitae-Revista De La Facultad De Quimica Farmaceutica* **2009**, *16*, 144.
(8) Larsson, C.; Rodahl, M.; Hook, F. *Anal. Chem.* **2003**, *75*, 5080.
(9) Yamaguchi, S.; Shimomura, T.; Tatsuma, T.; Oyama, N. *Anal. Chem.* **1993**, *65*, 1925.
(10) Zhao, J.; Zhang, X. Y.; Yonzon, C. R.; Haes, A. J.; Van Duyne, R. P. *Nanomedicine* **2006**, *1*, 219.
(11) Graneli, A.; Edvardsson, M.; Hook, F. *Chem. Phys. Chem.* **2004**, *5*, 729.
(12) Su, X. D.; Wu, Y. J.; Knoll, W. *Biosens. Bioelectron.* **2005**, *21*, 719.
(13) Pohanka, M. *Interdiscip. Toxicol.* **2009**, *2*, 52.
(14) Pohanka, M.; Jun, D.; Kalasz, H.; Kuca, K. *Protein Pept. Lett.* **2008**, *15*, 795.
(15) Schnell, J.; Dyson, H.; Wright, P. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 119.
(16) Skladal, P. *Food Technol. Biotechnol.* **1996**, *34*, 43.
(17) Sole, S.; Merkoci, A.; Alegret, S. *Crit. Rev. Anal. Chem.* **2003**, *33*, 127.
(18) Tseng, S. J.; Hsu, J. P. *J. Theor. Biol.* **1990**, *145*, 457.
(19) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*; Freeman, W. H., Ed.; Longman: Boston, 2004.
(20) Briggs, G. E.; Haldane, J. B. S. *Biochem. J.* **1925**, *19*, 339.

(21) Pesquero, N. C.; Pedroso, M. M.; Watanabe, A. M.; Goldman, M. H. S.; Faria, R. C.; Roque-Barreira, M. C.; Bueno, P. R. *Biosens. Bioelectron.* **2010**, *26*, 36−42.

(22) Sauerbrey, G. *Z. Phys.* **1959**, *155*, 206.

(23) Hegnerova, K.; Bockova, M.; Vaisocherova, H.; Kristofikova, Z.; Ricny, J.; Ripova, D.; Homola, J. *Sens. Actuators, B* **2009**, *139*, 69.

(24) Center, A. s. D. E. a. R. A. *Alzheimer's Disease Medications; A Service of the National Institute on Aging*; National Institutes of Health, U.S. Department of Health and Human Services: Washington, DC, 2009.

(25) Coelho, F.; Birks, J. Cochrane database of systematic reviews; 2001, CD001499. http://onlinelibrary.wiley.com/o/cochrane/clsysrev/articles/CD001499/frame.html.

(26) Giacobini, E. Cholinesterase inhibitors do more than inhibit cholinesterase. In *Alzheimer Disease: From Molecular Biology to Therapy*; Becker, R., Giacobini, E., Eds.; Birkhäuser: Boston, MA, 1996; p 187.

(27) Hsieh, B. C.; Matsumoto, K.; Cheng, T. J.; Yuu, G.; Chen, R. L. C. *J. Pharm. Biomed. Anal.* **2007**, *45*, 673.

(28) Valenti, P.; Rampa, A.; Bisi, A.; Andrisano, V.; Cavrini, V.; Fin, L.; Buriani, A.; Giusti, P. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2599.

(29) Hook, F.; Kasemo, B.; Nylander, T.; Fant, C.; Sott, K.; Elwing, H. *Anal. Chem.* **2001**, *73*, 5796.

JP106274M