Bull. Mater. Sci. (2020) 43:77
https://doi.org/10.1007/s12034-020-2055-2

AC impedance measurement for the enzyme kinetics of urea–urease system: a model for impedimetric biosensor

MOHANARANGAN SUNDARARAM[1], KUMAR JANAKIRAMAN[1], ANNAMALAI SENTHIL KUMAR[2], V LAKSHMINARAYANAN[3] and KRISHNAN SANKARAN[1,*]

[1]Centre for Biotechnology and Crystal Growth Centre, Anna University, Chennai 600025, India
[2]Nano and Bioelectrochemical Laboratory, Department of Chemistry, School of Advanced Sciences, Vellore Institute of Technology, Vellore 632014, India
[3]Soft Condensed Matter Laboratory, Raman Research Institute, Bangalore 560080, India
*Author for correspondence (ksankran@yahoo.com)

MS received 31 August 2019; accepted 27 November 2019

Abstract. The measurement of time evolution of electrochemical impedance enables enzymatic kinetic studies in real-time, and obviates the need of using additional reagents as in many popular spectroscopic methods. This can eventually lead to the development of enzyme biosensors. We have used the urea–urease system as a model for this study. The usage of a free enzyme (without any immobilization steps) in this work makes the technique very simple and unique for electrochemical measurement on urease. The impedance *vs*. time measurement of urease exhibits Michaelis–Menten (MM) behaviour with the MM constant (K_m) of 0.8 mM and maximum velocity (V_{max}) of 5000 ohms min^{-1}. This K_m value closely matched the one, which is obtained from the conventional colorimetric method (values). The enzyme kinetics was performed in a standard three-electrode system and reproduced in a fabricated mini electrochemical cell in an Eppendorf tube, which could pave the way for the development of impedimetric biosensors for a variety of enzyme systems, especially the ones for which spectrometric techniques cannot be readily applied.

Keywords. Urease; impedance *vs*. time measurement; enzyme biosensor; AC impedance.

1. Introduction

Biosensors are important for a variety of applications in various fields, such as environmental, pharmaceutical, industrial, medical, food and agriculture. It is gaining popularity because of its ease-of-use, sensitivity, specificity, accuracy, real-time measurement, rapidity and reliability for multiple analyses. A typical biosensor should be portable, provide rapid detection and should be of low cost. Transducer is the key to the novelty of the application along with the physicochemical property of an analyte [1]. It is also helpful in the enhancement of sensitivity, specificity, accuracy, precision, resolution and reproducibility. Biosensors are broadly classified into three types based on the transducer, optical or electrochemical or piezoelectric [2–4]. The electrochemical biosensors are more attractive and gaining popularity because of its feasibility, fidelity and robustness [4].

The signal transduction in biosensors using electrochemical impedance spectroscopy (EIS) is sensitive to femto molar concentrations of analytes [5]. EIS measures a charge transfer process at the electrode surface–solution interface [3]. The frequency-domain response (AC voltage) can give more information about the physical and chemical properties of a particular analyte (ionic substances) in EIS. In addition, some of EIS measurements do not require any labelling to monitor the analyte in real time [6]. Most of the impedimetric-based biosensors are operated with applied AC voltage over a wide range of frequency from 100 kHz to 1 MHz [7]. Some impedance measurements are based on the changes in the dielectric status of the cell membrane, which is measured at higher frequencies in the range of 100 kHz–20 MHz. The automated haematology analysers in clinical laboratories are based on impedance flow cytometry [8].

The change in the substrate or product concentration in a charge-based enzymatic reaction would lead to the impedance changes at the surface–solution interface. The real-time measurements of such enzymatic reactions are possible with impedance measurement as a function of time. The enzyme-based biosensing should have simple steps by eliminating immobilization of enzymes [9] and other labelling processes [5]. The latter in point-of-care detection device with active enzymes imposes serious limitations and it is a challenge in designing such sensors [5]. So, the direct measurement is desirable for the biosensor to be simple and effective.

The enzymes like alkaline phosphatase, esterase and urease catalyse charge-based enzymatic reactions by liberating the charged products like phosphate (PO_4^-), acetate (CH_3COO^-)

Published online: 21 February 2020

and ammonia (NH_4^+) ions in the solution. Therefore, the changes in the ionic concentrations are expected to have an effect on the measured impedance of the electrolyte solution during the course of the reaction [10]. Impedance technique has also been shown to be a promising method for on-the-spot detection of certain analytes [11].

In this work, we measure the impedance changes caused by the enzyme–substrate reaction within a short time after the commencement of the reaction. This is important, since in the study of MM kinetics, the velocity measurement at the initial stages (V_0) provides a more accurate computation of K_m value. Therefore, the proposed method is expected to provide a more reliable tool for the study of enzyme kinetics. By using the enzyme in soluble-free state, this method also avoids the surface immobilization of the enzyme as in the cases of previous reports based on impedance studies. Such a study is expected to realistically reflect the actual enzyme–substrate kinetics in real samples. The method is based on single frequency measurement, carefully chosen from the measured impedance spectra over a range of frequencies. Such a measurement is best suited for the development of hand-held device suitable for field applications.

In this work, the urease–urea reaction has been employed as a model system to follow the charge-based enzymatic reaction. The urea–urease reaction liberates ammonium ions in the solution during the reaction. The product concentration ($NH_4^+OH^-$) would increase during the course of reaction time resulting in change in impedance. The real-time measurement of urease kinetics was performed in the impedance–time measurement with specific applied AC voltage and frequency and showed that it was comparable to the results from the standard colorimetric method, but without the need for adding extraneous reagents.

2. Materials and methods

2.1 *Electrode and its pretreatment*

Platinum disc electrodes of 3 mm diameter were used as the working electrode and counter electrode along with the Ag/AgCl reference electrode for a standard three-electrode system.

2.2 *Electrochemical instrumentation*

All the electrochemical studies were carried out with CHI instruments (model 660C), electrochemical workstation (USA) for AC impedance, impedance–time and open circuit potential (OCP)–time measurements.

2.3 *Electrochemical cells*

The electrochemical experiments were conducted with a standard three-electrode system as well as a fabricated cell, which are depicted in figure 1a and b, respectively.

In the standard cell, two platinum electrodes of 3 mm diameter were used as working and counter electrodes, while the Ag/AgCl electrode acted as a reference electrode. The working and counter electrodes were sonicated successively in the dilute aqua regia, distilled water and finally in Millipore water. Urease assay was performed in the standard three-electrode system using 1 ml of reaction volume.

A fabricated electrochemical cell suitable for enzymatic analysis is shown in figure 1b. In a 2-ml Eppendorf tube, three pinholes were made for electrode insertion. Two platinum wires, one as working and the other as counter electrode and a silver wire acted as a reference electrode were bound to the

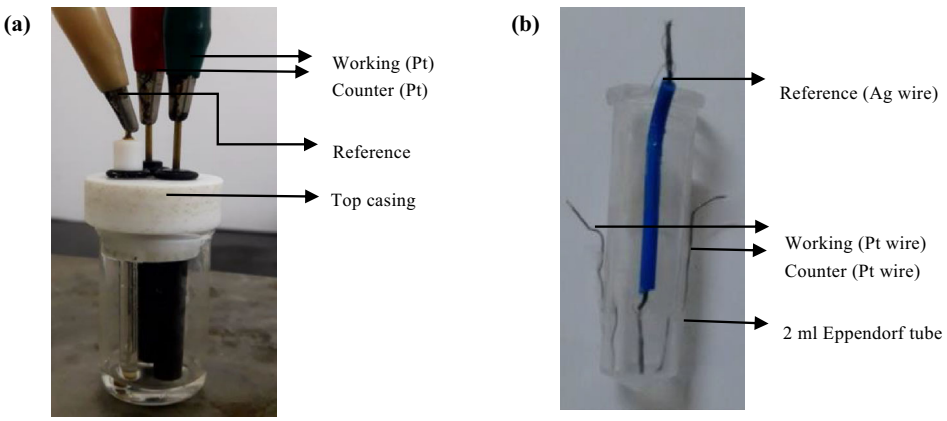

Figure 1. **(a)** Standard electrochemical cell with a platinum working and a counter electrode and Ag/AgCl as the reference electrode assembled in a top casing for measuring 1 ml of enzymatic reaction in a 10 ml of glass vessel. (**b**) Fabricated mini electrochemical cell in an Eppendorf tube of 2 ml volume to measure enzymatic reactions of 0.2 ml. Two Pt wires, as shown, served as the working and the counter electrodes and a silver wire (with sleeve) as the reference electrode.

outside wall of the cell by cellophane tape to ensure that inside, they remain equidistantly separated. Two-hundred microlitres of reaction volume was sufficient that the three electrodes were dipped to cover their maximum surface area.

2.4 *Chemicals*

Urease enzyme with specific activity of 200 U mg^{-1} extracted from Jack Bean, and other analytical grade chemicals such as phenol, sodium hydroxide and nitroprusside were purchased from Sisco Research Laboratory, India.

2.5 *Procedure for impedance measurement of urease reaction*

The OCP of the working electrode in the electrolyte containing the urease system was measured after attaining a stable value before performing the impedance study. The AC Impedance measurement was performed at this OCP with a frequency scan set at the range of $10-10^5$ Hz.

From the Z *vs.* log f plot, an appropriate single frequency at which the ionic conductivity dominates is chosen for the measurements of time evolution of impedance during the urea–urease reaction. This single frequency Z *vs.* t measurement was recorded immediately after the addition of urease. The reaction time was recorded from 0 to 120 s after the addition of enzyme. The above-mentioned procedure was followed for both the standard and fabricated electrochemical cells. The ΔZ of urease reaction was calculated for a time interval (Δt) of 30 s, after the first 30 s has elapsed from the addition of urea, so that the equilibrium is reached. The rate of change of impedance is then ($\Delta Z/\Delta t$) is plotted against concentration of urea to obtain the kinetic data.

2.6 *Urease assay by impedance–time measurement*

Rate of change of impedance *vs*. substrate concentration was measured in standard 3-electrode system and plotted similar to the typical rate of reaction *vs*. substrate concentration plot (Michaelis–Menten). Urease activity was measured as impedance change per 30 s at different urea concentrations from 0 to 5 mM (with an incremental addition of 0.5 mM substrate) in 5 mM Tris-HCl, pH 7.2, containing 40 μg ml^{-1} of enzyme in an assay volume of 1 ml. For inactive enzyme study, the urease at 1 mg ml^{-1} in the above buffer was inactivated by keeping it in a dry bath at 95°C for 1 h. For observing the increase in velocity as a function of enzyme concentration, urease concentration was increased from 0 to 50 μg ml^{-1} in increments of 10 mg ml^{-1}; concentration of urea was maintained at 3 mM. Only active urease was assayed in the fabricated cell maintaining the same reaction conditions except the assay volume was 200 ml.

2.7 *Colorimetric urease assay for validating impedance assay*

To validate the impedance method, the standard colorimetric assay was performed alongside. The micro version of Berthelot method [12] of urease assay involved incubating for 10 min, 10 μg $ml^{-1}$of urease in 200 ml of 20 mM Tris-HCl buffer, pH 7.2 containing urea in a microtitre plate well at room temperature. Addition of 100 μl Berthelot reagent (60 mM sodium phenate, 0.5% w/v of sodium hypochloride and 0.1% of sodium nitroprusside) to the reaction mix after the incubation resulted in the development of blue colour in 10 min. Optical density (OD) at 630 nm was read in a commercial microplate reader. From the standard graph of OD_{630nm} *vs*. ammonium ion (product) concentration, the amount of product released was determined and the velocity of the reaction was calculated. Kinetic plot of velocity *vs*. substrate concentration was then compared with the rate of impedance change *vs*. substrate concentration. K_m (substrate concentration at half maximal velocity) and V_{max} (maximal velocity) were calculated for both the methods by standard enzyme kinetic calculations. Inactive enzyme was also assayed by the colorimetric method and the results compared with the impedance measurement.

3. Results and discussion

3.1 *Impedance measurement for following the urea–urease reaction*

The OCP measured between the working and reference electrodes in the standard and fabricated cells was found to be 0.23 and 0.2 V, respectively. The impedance measurements were carried out at this OCP in the frequency range between 0.1 Hz and 100 kHz. Figure 2a shows the Bode plots of the urea–urease reaction at 0 and 5 mM concentrations of urea in the electrolyte. The total impedance decreases at low frequency and asymptotically reaches a steady state at high frequencies. This steady state region corresponds predominantly due to the solution resistance arising solely from the ionic conductivity of the electrolytic medium. There is a significant decrease in the total impedance values measured at the high frequency region on the addition of 5 mM urea in urease as shown in figure 2b. This is due to the generation of charged reaction products of NH_4^+ and Cl^-, which leads to an increase in the conductivity of the medium, thereby decreasing the overall measured total impedance. Jun Li *et al* [13] demonstrated that the phosphorylation and dephosphorylation reactions of kinase and alkaline phosphatase in real-time electrochemical impedance measurement are similar to the above concept.

The changes in the values of the measured total impedance at higher frequency regions, as seen in figure 2b after the addition of urea can be correlated with the generation of charged reaction products. In other words, if the time evolution of

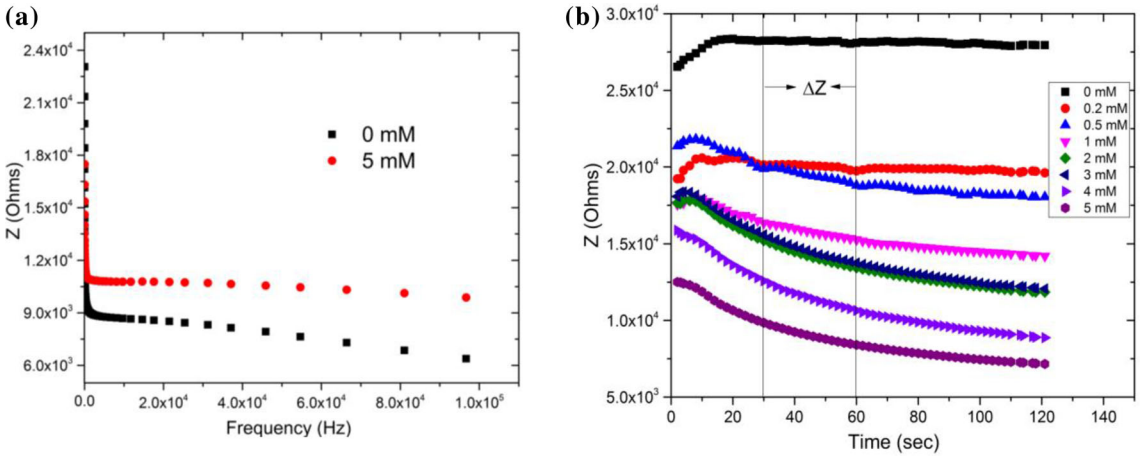

Figure 2. (**a**) Bode plot for the urease reaction at 0 and 5 mM urea in the standard cell. The impedance studies were carried out at OCP. (**b**) Impedance–time plots for urease were obtained at a frequency of 100 kHz for enzymatic reactions with 0–5 mM urea, 40 μg ml^{-1} urease in 5 mM Tris-HCl, pH 7.2 in a volume of 1 ml. The reaction was initiated with the addition of the enzyme. The ΔZ values are the difference of $Z_{60\text{th s}}$ and $Z_{30\text{th s}}$ for each (urea) during the urease reaction.

the total impedance is followed at any suitable frequency, the reaction velocity can be measured at any instance of time. We have used this concept to study the reaction kinetics of urea–urease reaction at 100 kHz, where the difference in the total impedance between 0 and 5 mM addition of urea is maximum.

It can be noted that the change in the impedance is rapid at initial stages of the reaction, which asymptotically reaches a constant value. After the first 30 s of the commencement of the reaction in order for the reactants to equilibrate, the impedance changes for the next 30 s have been considered for measuring the rate of hydrolysis. A plot of the measured $(\Delta Z/\Delta t)$ *vs.* concentration of urea can be used to obtain the Michaelis–Menten plot for the urea–urease enzymatic reaction.

3.2 *Michaelis–Menten plot for real-time measurement of urease reaction*

The urease assay was performed with standard electrochemical cell as well as the fabricated cells. The values of $(\Delta Z/\Delta t)$ are plotted against the concentration of urea with fixed urease concentration in the solution. The MM plots of urease assay based on the impedance measurements are shown in figure 3a and c. The behaviour is similar to colorimetric measurement of urease assay, which is depicted in figure 3b. The $\Delta Z/\Delta t$ values plateau at concentrations of urea saturating the enzyme both in standard and fabricated cells, which is indicative of the maximum velocity (standard cell — $V_{\max} = 65$ ohms s^{-1}, fabricated cell — $V_{\max} = 13$ ohms s^{-1}). From the curve fitting for the MM equation, the K_{m} values of the standard and fabricated cells are calculated to be 0.82 and 0.97 Mm, respectively, from the plot. The K_{m} values of urease assay measured from both the cells were in reasonable agreement, considering different cell dimensions of the standard and fabricated cells.

The inactive urease assay shows insignificant changes in $\Delta Z/\Delta t$ values for the same concentration of urease in active urease. It is therefore, clear that urea concentration has significant effect on $\Delta Z/\Delta t$ values only for the enzymatic reaction. In further evidence, figure 4 shows a linear increase in $\Delta Z/\Delta t$ for the increase in urease concentration from 0−50 μg ml^{-1}.

3.3 *Comparative analysis for impedance measurement with the colorimetric method*

The comparative analysis of impedance measurement with the colorimetric method is presented in table 1. Both the methods are compared for the enzyme parameters, such as K_{m} values and specific activity of urease. The impedance and colorimetric measurements show the similar MM kinetics as depicted in figure 3. The K_{m} values measured using colorimeteric (0.9 mM) and impedance methods (0.82 for standard cell and 0.97 mM for the fabricated cell) agree with each other closely. The K_{m} values of urease as reported in literature vary widely and are in the range of 0.5–3.2 mM [14,15]. In addition, the colorimetrically measured specific activity of 179 units mg^{-1} of the commercial urease preparation matched with the specified value of 200 units mg^{-1}. However, in the case of impedance method, velocity in terms of product formed per unit time cannot be determined, but there is maximal impedance change per unit time for a given enzyme concentration. Since the impedance is a property of reaction milieu, rather than its individual components, its maximum difference is the maximum change in the reactant compositions or reaction that had happened in the time taken. So, $\Delta Z_{\max}/\Delta t$ for an enzyme reaction will be a constant like $V_{\max}$, but that of the reaction composition under the conditions used. The specific activity of an enzyme, $\Delta Z/\Delta t$ per mg was found to be a constant from

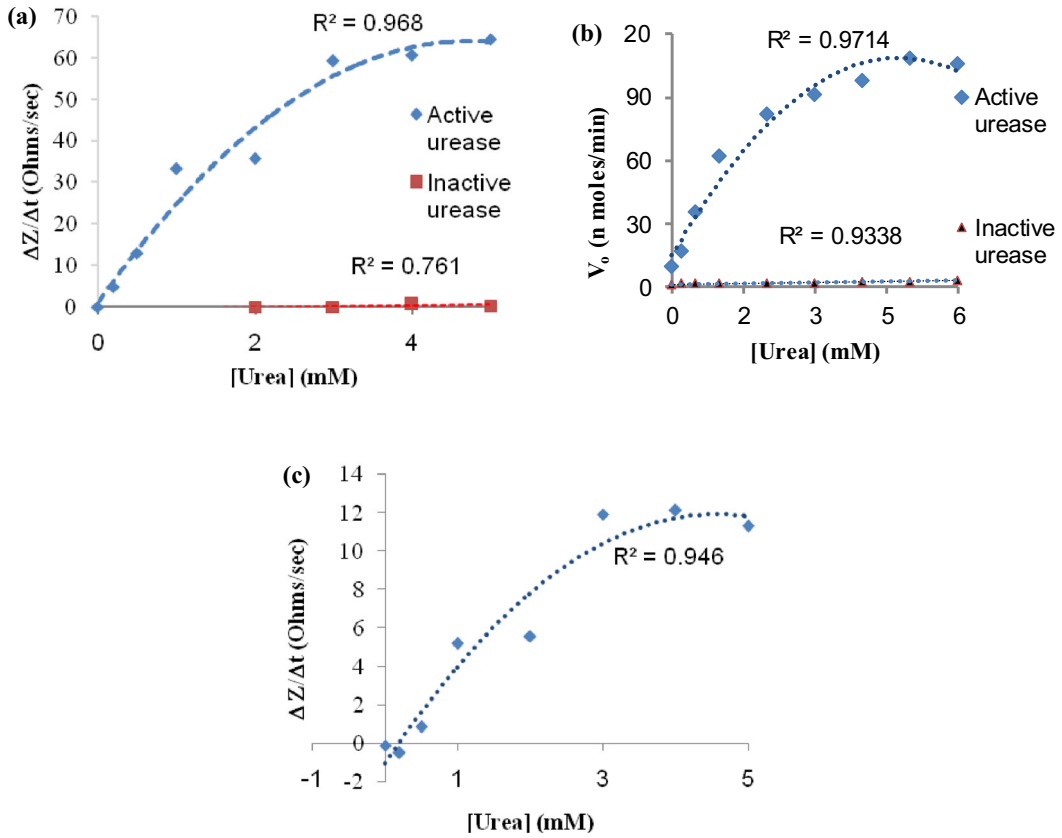

Figure 3. (**a**) MM plot derived from impedance–time measurement (figure 2b) for urease assay (0–5 mM urea with 40 μg ml^{-1} urease) in the standard three-electrode system. The same procedure was followed for the inactive urease (heat-treated at 95°C for 1 h) assay. (**b**) MM plot obtained from the standard colorimetric assay of urease (0–5 mM urea with 10 μg ml^{-1} urease). The same procedure for the inactive urease (heat treated at 95°C for 1 h). (**c**) MM plot obtained from the impedance–time measurements (figure 2b) for urease assays (0–5 mM urea with 40 μg ml^{-1} urease), conducted at 100 kHz in fabricated three-electrode system.

the velocity *vs*. enzyme concentration plot as 45.6 ohms s^{-1} mg^{-1} within 10% error.

The primary purpose of the work reported here is limited to the developing impedance measurement for the study of enzyme kinetics as a better alternative to conventional spectrophotometric method. Towards this goal, we have carried out the impedance measurements within the typical concentration range used for MM plot for urea–urease reaction. Hence, it has the potential to measure even at much lower concentrations. The use of Pt and Ag wires as electrode materials have the advantage of surface renewal and reusability, providing pristine surface (by following certain cleaning protocols) essential for reliable enzyme kinetic studies with a variety of chemical compositions. Use of screen-printed electrodes, carbon-based electrodes is not recommended for such a study as the surface anchoring of the enzyme on the functional groups on the carbon-based electrodes can inhibit the catalytic active sites. Free enzyme provides homogeneous distribution with more catalytic sites and substrate accessibility than surface-immobilized enzymes. It is therefore, more

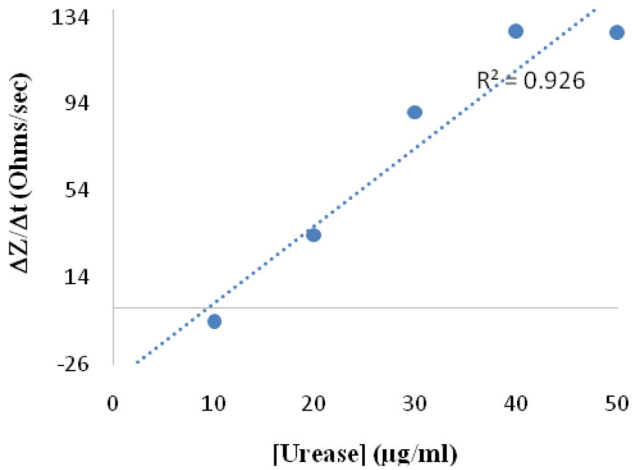

Figure 4. Impedance–time measurement as a function of urease concentration from 0 to 50 μg ml^{-1} measured at 100 kHz. The experiment was performed in 3 mM urea contained in 5 mM Tris-HCl, pH 7.2 in an assay volume of 1 ml using the standard three-electrode system.

Table 1. Enzyme parameters obtained from impedance and colorimetric measurements of urease.

Enzyme parameter	Enzyme system	Colorimetric method	Impedance method	
			Standard three electrode system	Fabricated electrode system
K_m values (mM)	Urease	0.9	0.8	1.0
Specific activity[a] (units mg^{-1})	Urease	179	—	—
Specific activity[b] (Ohms s^{-1} mg^{-1})	Urease	—	45.6	—

[a]Measured from colorimetric methods and [b]measured from AC impedance method.

reliable indicator of the enzyme–substrate reaction. The formation of charged ammonium ions as product alters both the interfacial and bulk charges in the reaction mixture leading to the changes in the measured impedance.

4. Conclusion

A simple and direct method based on impedance measurement without any intervening immobilization step has been proposed for the study of charge-based enzymatic kinetics with urea–urease as a model system. To the best of our knowledge, this is the first time that the impedance changes during the enzyme–substrate reaction are measured within a short time after the commencement of the reaction (within the first 60 s under the initial velocity condition). In other words, the slope is measured for the changes during the 30 s after the first 30 s have elapsed for the equilibrium to be attained. It may be pointed out that in the study of MM kinetics, velocity measurement at the initial stages (V_0) provides more accurate results and therefore, the proposed method provides a more reliable tool for the study of enzyme kinetics.

The AC impedance measurement of the enzymatic reaction shows significant promise for real-time analysis. The Michaelis–Menten constant obtained by impedance measurements is in good agreement with the spectrophotometric measurement for the urea–urease reaction. Apart from detecting enzymatic reactions rapidly, this method is useful for kinetics without added reagents, a significant advantage for enzymes that are measured indirectly and for systems for which spectrometric measurements are not possible. In conclusion, AC impedance-time measurement can be used in the development of cost-effective and reliable enzyme biosensor for a reagent-less and real-time measurement of analytes.

Acknowledgements

We acknowledge the Department of Science and Technology, Technology Development Programme (DST/TSG/ME/2010/62), Scheme for the financial support. We thank Centre for Biotechnology, RRI Bangalore and VIT Vellore for providing the lab facilities.

References

[1] Goode J A, Rushworth J V and Millner P A 2015 *Langmuir* **31** 6267
[2] Shagun M, Abhishek V, Naveen T and Vivek K 2017 *IJARIIE-ISSN(O)* **3** 3639
[3] Pratima R S, Ajeet K, Agrawal V V and Malhotra B D 2011 *NPG Asia Mater.* **3** 17
[4] Tanu B 2015 *Int. J. Adv. Res. Sci. Eng. Tech.* **6** 36
[5] Jonathan S D and Pourmanda N 2007 *Electroanalysis* **19** 1239
[6] Asal M, Özen Ö, Sahinler M and Polatoglu I 2018 *Sensors (Basel)* **18** 1
[7] Hammond J L, Formisano N, Estrela P, Carrara S and Tkac J 2016 *Essays Biochem.* **60** 69
[8] Cheung K C, Berardino M, Schade K G, Hebeisen M, Pierzchalski A, Bocsi J *et al* 2010 *Cytometry A* **77** 648
[9] Sassolas A, Blum L J and Leca-Bouvier B D 2012 *Biotechnol. Adv.* **30** 489
[10] Narendran S and Ramasamy R P 2013 *J. Microbial. Biochem. Technol.* **S6** 4
[11] Wang Y, Zunzhong Y and Yibin Y 2012 *Sensors* **12** 3449
[12] Paliwal D K and Randhawa H S 1977 *J. Appl. Environ. Microbiol.* **33** 219
[13] Yien L, Lateef S, Jianwei L, Duy H and Jun L 2012 *Anal. Chim. Acta* **744** 45
[14] Selvamurugan C, Lavanya A and Sivasankar B 2007 *J. Sci. Ind. Res.* **66** 655
[15] Robert L B, Edwin C W and Burt Z 1969 *Biochem.* **8** 1984