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Research Article

Molecularly Imprinted Polymer Based Biosensor For Choline

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ABSTRACT

Biosensors are systems that can perform quantitative and/or qualitative analysis of substances in liquid or gas environment through their biological recognition sites and transform the acquired data into detectable signals. Biosensors are able to detect physical changes (i.e. as density, mass concentration, etc.) by means of recognition sites and correlate them with electrical or optical quantities (i.e. current, voltage and impedance). In this study, three molecularly imprinted pencil graphite electrodes with differing numbers of choline recognition sites, at E-1 M, E-3 M and E-5 M concentration, were used as electrochemical biosensors. An increase in choline receptor concentration on the electrode surface was expected to correlate with an increase in PGE surface bound choline and thus lead to electrical changes. The study was conducted in a three-electrode cell with Ag/AgCl as the reference electrode, platinum wire as the counter electrode and PGE as the working electrode. Cyclic voltammetry and electrochemical impedance measurements were conducted in 10 mM phosphate buffer solution containing 5mM $K_3[FeCN_6]^{-3/-4}$ redox pair. As expected, as increasing amount of choline was bound to the complementary recognition sites on choline imprinted electrodes, a correlating change in current, voltage and impedance was observed. The dynamic detection range for choline expanded as the choline concentration imprinted on the electrodes increased. Using the E-1 M PGE electrode, 72 pM limit of detection, up to 7.2 nM limit of linearity was attained.

Keywords: *Biosensor, electrochemical impedance spectroscopy, choline, molecularly imprinted polymer, PGE electrode.*

Kolin Tespiti İçin Moleküler Baskilama Tabanli Biyosensör Geliştirilmesi

ÖZET

Biyolojik sensörün kısaltması olarak kullanılan biyosensörler, maddelerin sıvı ya da gaz ortamda nicel veya nitel tayinini sahip olduğu biyolojik tanıma bölgeleri sayesinde yapabilen ve elde ettiği verileri tespit edilebilir sinyallere çeviren sistemlerdir. Biyosensörler, uygun tanıma bölgeleri aracılığıyla fiziksel değişiklikleri (yoğunluk, kütle, derişim vb.) tespit edebilmekte ve bunları elektriksel veya optik büyüklüklerle (akım, gerilim, empedans vb.) ilişkilendirmektedir. Bu çalışmada, E-1 M, E-3 M ve E-5 M olmak üzere 3 farklı derişimde moleküler baskılanmış, farklı sayıda kolin tanıma bölgelerine sahip, kalem grafit elektrotlar, elektrokimyasal biyosensörler olarak kullanılmıştır. Elektrot yüzeyindeki kolin reseptörü derişimindeki artışın, elektrod yüzeyine

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bağlı kolindeki artışla ilişkili olması ve dolayısıyla elektriksel değişikliklere yol açması beklenmektedir. Çalışma, üç elektrotlu hücrede, referans elektrot olarak Ag/AgCl, karşı elektrot olarak platin tel ve çalışma elektrotu olarak PGE kullanılarak gerçekleştirilmiştir. Elektrotların açık hücre potansiyeli, dönüşümsel voltametri ve elektrokimyasal empedans ölçümleri, 5mM $K_3[FeCN_6]^{-3/-4}$ redoks çifti içeren 10 mM fosfat tampon çözeltisi içerisinde alınmıştır. Çözelti içerisindeki kolinin, kolin baskılanmış elektrodlar üzerindeki tamamlayıcı tanıma alanlarına bağlanmasıyla, elektrodlarda beklenen akım, voltaj ve empedans değişimleri gözlenmiştir. Baskılanan molekül derişiminin artışıyla bağıntılı olarak, tespit aralığında da bir artış gözlenmiştir. Sonuç olarak, E-1 M kolin baskılanan PGE, 7.2 nM-72 pM tespit aralığındaki kolin konsantrasyonunda en yüksek farklılaşmayı göstermiştir.

Anahtar Kelimeler: *Biyosensör, elektrokimyasal empedans spektroskopisi, kolin, moleküler baskılama, PGE elektrot*

I. INTRODUCTION

Biosensors are systems that detect changes in the concentration, density or amount of a given analyte and measure biological or chemical reactions by generating electrical signals proportional to these changes [1]. Biosensors can be used in many areas, especially in the field of health, where they are commonly used in the detection of diseases, as disease-causing viruses, bacteria, the amount of harmful substances or molecules used as biological markers. Attachment of microorganisms, enzymes, antibodies, nucleic acids and biomolecules generate correlating signals, which indicate presence and quantity of molecules enabling qualitative and/or quantitative identification. Thus, molecular recognition capability of biosensors increases the access to medical diagnosis and enables preliminary testing prior to extensive hospital laboratory examination.

In electrochemical sensors, the response of electrodes to variations in analyte concentration may be detected as a correlating change in current, voltage and impedance. As selectivity is a critical element in biosensor design, electrochemical biosensor selectivity may be increased using the molecular imprinting method, where molecule-specific recognition sites on the electrodes are created, allowing the molecule to attach to those regions. Using biomolecules even at low concentrations, highly selective and specific recognition sites for amino acids, hormones, enzymes, salts, vitamins and biomarkers, etc. may be formed on molecularly imprinted polymer (MIP) based biosensors [2-5]. Thus, artificial receptors may be obtained by forming molecule-specific recognition sites to be analyzed on pencil graphite electrodes (PGEs) using the MIP method, which involves polymerization on the surface of the electrodes using the template molecule as a stamp, removal of the template molecule and finally creating specific recognition sites for the template molecule to be analyzed [6-11].

Electrochemical biosensors may be modified using MIP for the detection of small molecules, specific metabolites observed in disease progress. Choline has been classified as a precursor molecule in the course of some diseases such as Alzheimer's, Parkinson's, prostate and pancreatic cancers, some liver and lung diseases and autism [12-17]. Choline, an essential small molecule of cellular membrane, a Vitamin B derivative with blood serum level of 7 μmol/L [12] emerges as a potential small molecule used as an early stage cancer biomarker in screening tests. Therefore, serum choline level appears as a parameter to be used in disease diagnosis and prognosis.

In this study, cyclic voltammetry and electrochemical impedance measurements were conducted by choline receptors formed on polymer imprinted pencil graphite electrodes. In this study, with the binding of choline to MIP-PGE electrode [18], a decrease in conductivity (based on I-V characteristics of the cyclic voltammetry (CV) graph) and an increase in impedance (based on imaginary and real impedance plots of the electrochemical impedance spectroscopy (EIS) graph) were expected.

II. MATERIALS AND METHODS

A. MATERIALS

In this study, a total of twenty-five PGEs (Tombow, 0.9) were used. All PGEs were initially anodized in acetic buffer solution (ABS, 0.1 N acetic acid, 0.1 N sodium acetate, 20 mM sodium chloride pH 4.8) with a voltage of +1.4 V. The electrodes were then divided into five groups. The bare electrode group PGEs (B-PGEs); the control group PGEs (C-PGEs) were coated with polymer without choline; and the three experimental groups contained choline at E-5 M (E-5PGE), E-3 M (E-3PGE) and E-1 M (E-1PGE) concentration imprinted on the PGEs.

The molecular imprinting process was conducted following Ref [19], where choline was used as the template molecule, methacryl amido glutamic acid (MAGA) (Nanoreg, USA) as the functional monomer, ethylene glycol dimethacrylate (EGDMA) (Aldrich, USA) as cross linker and azobisisobutyronitrile (AIBN) (Fluka, Buchs-Switzerland) as the initiator of polymerization conducted under ultraviolet (UV) light at 365 nm. EGDMA, MAGA, 2 mg of AIBN and choline (at E-5M, E-3 M, and E-1 M concentration) were combined in an eppendorf tube. The anodized electrodes were immersed in this solution for two minutes, removed and allowed to polymerize for 20 minutes under the UV light to activate the AIBN. After polymerization, choline was removed by immersing the electrodes in 0.1 M HCl solution for ten minutes in order to form choline-specific sites and finally the electrodes were washed twice by immersing in deionized water for five minutes. CV and EIS analyses were conducted in 10 mM phosphate buffer solution (PBS) (1.37 M sodium chloride (NaCl), 27 mM potassium chloride (KCl), 100 mM disodium hydrogen phosphate (Na_2HPO_4), 18 mM potassium dihydrogen phosphate (KH_2PO_4), pH 7.4,) containing 5 mM potassium ferricyanide, $K_3[Fe(CN)_6]$ (RdxPBS) redox pair [20]. Choline (Sigma-Aldrich, USA) solutions were prepared at five different concentrations, ranging from the most diluted (72 pM, CE-11) to the most concentrated (0.72 µM, CE-7) as CE-11, CE-10, CE-9, CE-8 and CE-7. All solutions were prepared using analytical grade chemicals.

B. INSTRUMENTS

Electrochemical analysis was performed using the ZIVE SP2 Potentiostat/Galvonostat Reference 600 (South Korea) supported by the ZMAN EIS Data Analysis software. Measurements were conducted using a three-electrode system; the Ag/AgCl reference electrode, platinum wire (counter electrode) and PGE (working electrode).

C. ELECTROCHEMICAL MEASUREMENTS

Open cell potential (OCP) for each electrode was measured until the voltage was stabilized in 100 ml of RdxPBS, the standard volume used during this study. The initial CV and EIS measurements were carried out in RdxPBS without choline. CV measurements in RdxPBS were obtained in the -500/+ 500 mV range (scan rate: 50 mV/s, step size: 20 mV). I-V curves were plotted during anodic and cathodic potential changes. The peak oxidative current was obtained using the positive increase in potential of $Fe(CN)_6^{-4}$ and the peak reductive current was obtained using the negative increase in potential of $Fe(CN)_6^{-3}$.

$$Fe(CN)_6^{-4} \rightarrow Fe(CN)_6^{-3} + e^- \quad (1)$$

$$Fe(CN)_6^{-3} + e^- \rightarrow Fe(CN)_6^{-4} \quad (2)$$

Impedance graphs of PGEs in RdxPBS were obtained by applying a potential of +10 mV in the range of 10^{6} - 10^{-2} Hz. CV and EIS response of the electrode to increasing choline concentration in solutions from CE-11 to CE-7 range was investigated.

III. RESULTS AND DISCUSSION

A. PENCIL GRAPHITE ELECTRODES AND CHOLINE SOLUTIONS

All PGEs were anodized in 100 ml ABS at +1.4 V until the current was stabilized. At the end of the anodization process, PGE conductivity in the range of 15 µA-40 µA was attained. Electrodes prepared at three different imprinting concentrations as E-5 M (E5-PGE), E-3 M (E3-PGE) and E-1 M (E1-PGE), the bare electrode group (B-PGE) and the control group (C-PGE) were characterized using the RdxPBS solution containing choline (RdxPBS-choline) at varying concentrations ranging from 10^{-11} M to 10^{-7} M (respectively named as CE-11, CE-10, CE-9, CE-8 and CE-7). The EIS analysis was then used to electrochemically demonstrate choline-choline receptor binding in RdxPBS-choline solutions as a change in impedance.

B. THE BARE ELECTRODE GROUP

There were no recognition sites on the surface of these bare electrodes; therefore, added choline was not bound to the electrode. An increase in solution choline content was expected to lead to an increase in solution conductivity, displaying an increase in current in CV graphs as well as a decrease in impedance in EIS graphs of B-PGE (Fig. 2).

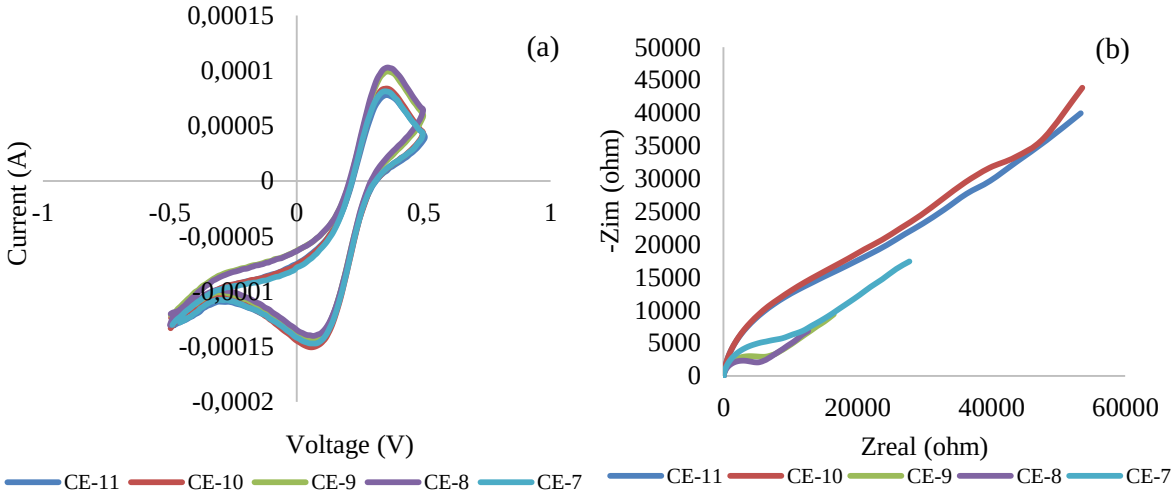

Figure 2: *CV (a) and EIS (b) graphs of bare electrode group, B-PGE.*

C. THE CONTROL GROUP

The control group electrodes with a plain polymer coating lacked choline recognition sites. Thus, an increase in conductivity and a decrease in impedance were expected. The CV and EIS electrochemical analyses results of the control group (C-PGE) electrodes in RdxPBS with varying amounts of choline are shown in Fig. 3.

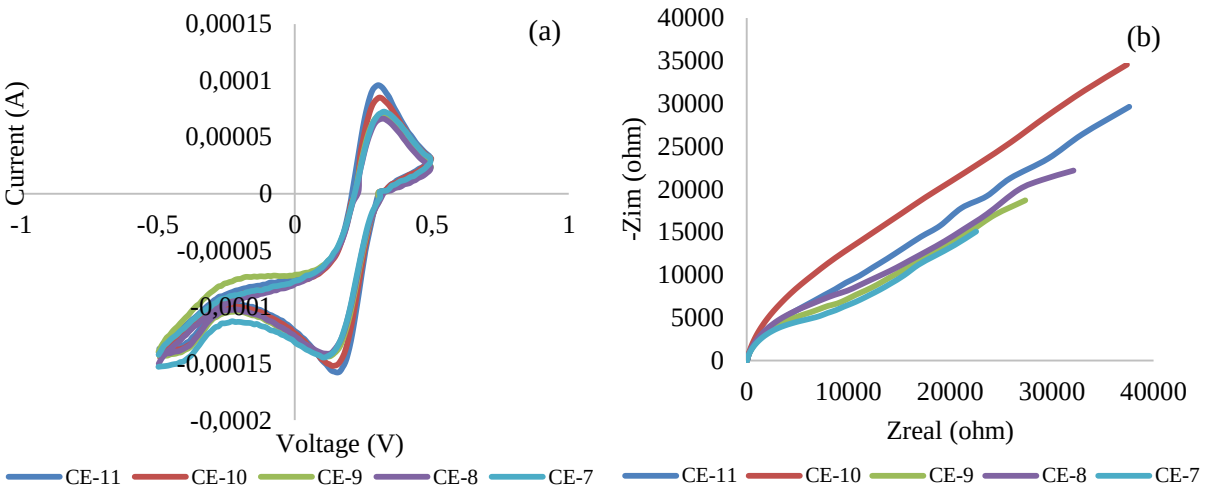

Figure 3: *CV (a) and EIS (b) graphs of the control group, C-PGE.*

D. EXPERIMENTAL GROUPS

The experimental polymer coated groups, containing different numbers of choline recognition sites were expected to display a reduction in current and an increase in impedance as choline was expected to bind to the recognition sites, reducing solution conductivity and thus creating a capacitive layer on the electrode.

E-5PGE: The CV (Fig. 4a) and EIS (Fig. 4b) graphs of the E-5PGE in RdxPBS indicate that impedance due to CE-11 addition can be distinguished from the overlapping CE-10, CE-9, CE-8 and CE-7 plots. After the addition of CE-10, bound choline saturating the recognition sites on electrode surface has thus formed a layer, which has reduced conductivity (Fig. 4a) while increasing impedance (Fig. 4b).

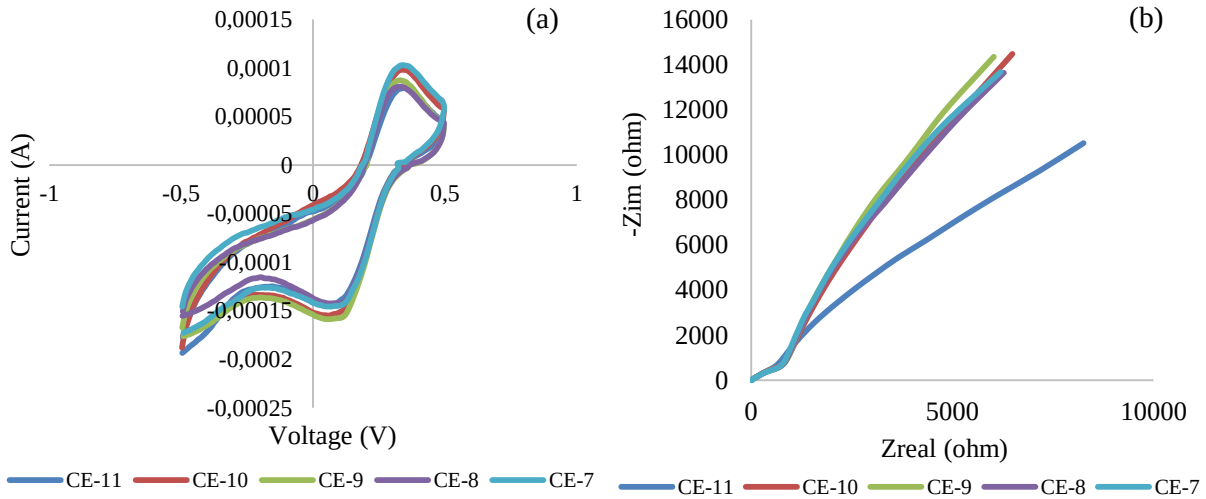

Figure 4: *CV (a) and EIS (b) graphs of E-5PGE.*

E-3PGE: The CV (Fig. 5a) and EIS (Fig. 5b) responses of the E3-PGE, with a higher number of choline binding sites on its surface than E-5PGE, and thus enabling a higher amount of choline to bind, indicate a distinct separation of CE-11, CE-10 and CE-9 choline levels (Fig. 5b). Although an increase in impedance from CE-11 to CE-9 is observed, the overlapping of CE-9 to CE-7 choline plots indicate saturation of choline binding sites on the E-3PGE electrode with the addition of CE-9 choline.

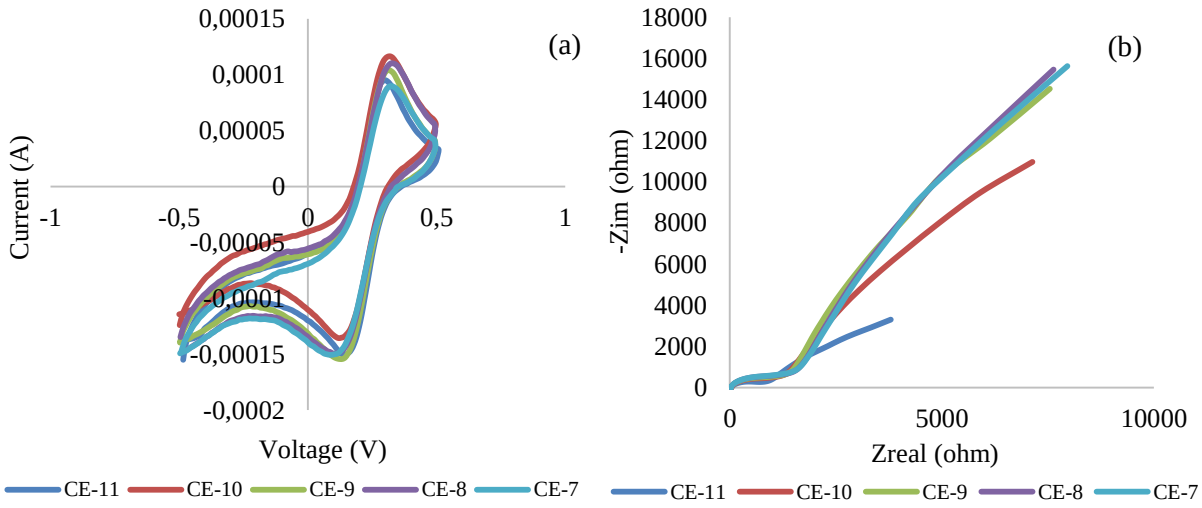

Figure 5: *CV (a) and EIS (b) graphs of E-3PGE.*

E-1PGE: The CV (Fig. 6a) and EIS (Fig. 6b) response of the E-1PGE, which has the highest concentration of imprinted choline indicate that choline levels ranging from CE-11 to CE-9 can be readily distinguished, while displaying a slight separation between CE-9 and CE-8. The overlapping EIS plots of the CE-8 and CE-7 solutions indicate saturation of the E-1PGE to choline at this concentration.

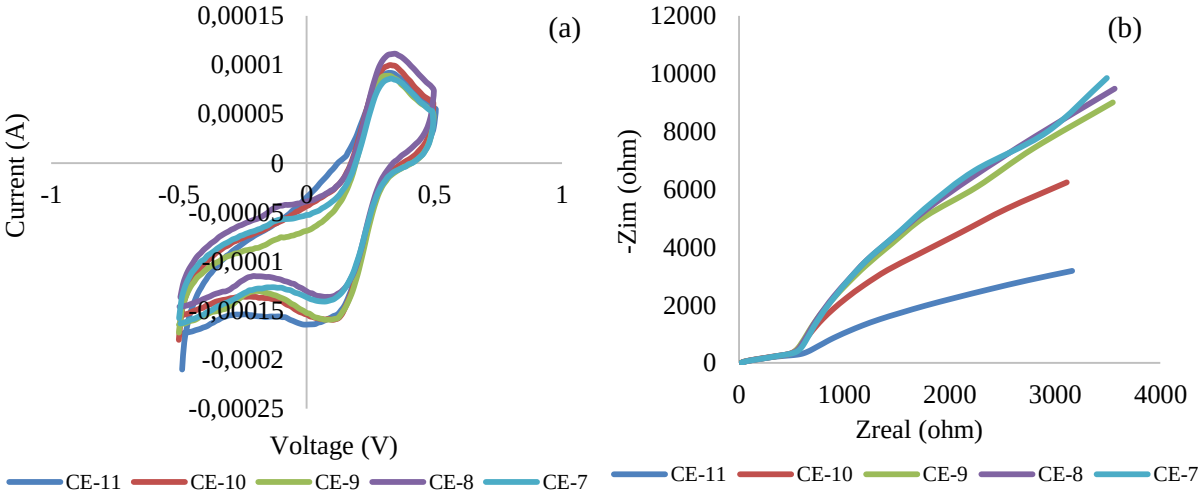

Figure 6: *CV (a) and EIS (b) graphs of E-1PGE.*

Analysis of the results of E-5PGE, E-3PGE and E-1PGE choline imprinted working electrodes in redox phosphate buffer solution (RdxPBS) using five different choline concentrations (72 pM (CE-11), 720 pM (CE-10), 7.2 nM (CE-9), 72 nM (CE-8) and 0.72 µM (CE-7)) indicate that the electrodes have responded to the increase in choline levels with a correlating increase in impedance as displayed by separation of CE solution EIS curves. As a result, an increase in choline concentration in molecularly imprinted binding sites on PGEs increased the dynamic range for choline especially at frequencies between 0.01-0.1 Hz. Thus, using the E-1PGE, 72 pM limit of detection and up to 7.2 nM limit of linearity was attained. Figure 7 shows the calibration curve of the E-1PGE using the CE-11 - CE-9 range of choline concentration. At this dynamic range, the differential impedance ratios vs. the logarithm of choline concentration indicates a linear change, which was used as a calibration curve (Fig. 7).

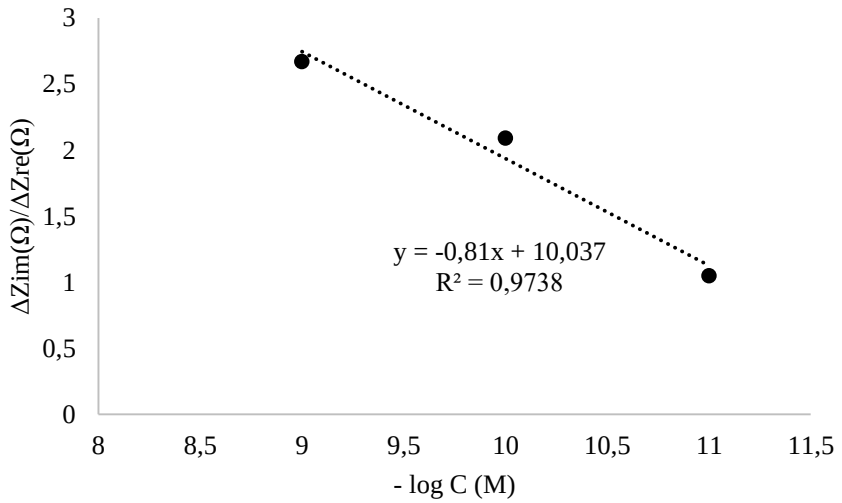

Figure 7: *Calibration curve of E-1PGE electrode in the CE-11 to CE-9 dynamic range.*

In this study, pencil graphite electrodes were used due to their high chemical and mechanical stability. PGEs are commonly used in many sensor applications as they are cheap, durable, stable, easily modified and reusable [21]. With an approximately 0.255 cm^2 active electrode surface area, they can be used in the analysis low concentrations of small volume samples. The graphite and polymer content of the PGEs, provide them different structural, chemical and physical properties, which are modified by chemical means as demonstrated in this study: the electrode surface was oxidized during the anodization process [21], limiting the current conducted through the electrode. Anodization was used to increase the electrode surface area within the limit of standardization of these electrodes. In quantitative CV analysis, the PGE electrode diameter appears as a factor in electron transfer rate and signal amplitude. In this study, in polymer coated electrodes, the electron transfer rate of the redox pair was reduced, leading to low anodic and cathodic current observed in CV graphs. In coated choline PGEs, because of alteration in load transfer resistance, oxidative and reductive peak current was reduced [22,23], showing that choline concentration is inversely related to the anodic and cathodic current conducted. An increase in choline concentration leads to a decrease in electron transfer rate of the redox pair and thus to an increase in impedance as displayed in EIS results. The fact that the impedance does not increase after a choline concentration has been reached indicates saturation of choline binding sites on the electrode at that concentration.

IV. CONCLUSION

In this study, the response of pencil graphite electrodes prepared by molecular imprinting method was investigated in phosphate buffer solution using various choline concentrations. EIS and CV analysis results indicated that the separation potential of choline imprinted PGEs increased with concentration of choline used to prepare the molecularly imprinted polymer at low frequencies. Using the E-1PGE, 72 pM limit of detection and up to 7.2 nM limit of linearity was attained. In the future, studies will be conducted to extend both the limit of detection and the dynamic range of the choline PGE by increasing the number of choline specific recognition sites imprinted on PGEs and to test these electrodes using a wider concentration range of choline solutions. In future studies, the physical and chemical properties of the electrodes will be modified in light of new data to enhance specificity, sensitivity and dynamic range.

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