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Detection of *Janus Kinase 2* gene single point mutation in real samples with electrochemical DNA biosensor

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ABSTRACT

Janus Kinase 2 (*JAK2*) gene single point mutations, which have been reported to be associated with myeloproliferative disorders, are usually detected through conventional methods such as melting curve assays, allele-specific and quantitative Polymerase Chain Reactions (PCRs). Herein, an electrochemical biosensor for the detection of a Guanine (G) to Thymine (T) transversion at nucleotide position 1849 of the *JAK2* gene was reported. Due to clinical importance of this mutation, easy and sensitive tests are needed to be developed. Our aim was to design a biosensor system that is capable of detecting the mutation within less than 1 h with high sensitivity. For these purposes, an electrochemical sensing system was developed based on detecting hybridization. Hybridization between probe and its target and discrimination of single point mutation was investigated by monitoring guanine oxidation signals observed at +1.0 V with Differential Pulse Voltammetry (DPV) by using synthetic oligonucleotides and Polymerase Chain Reaction (PCR) amplicons. Hybridization between probe and PCR amplicons was also determined with Electrochemical Impedance Spectroscopy (EIS). We successfully detect hybridization first in synthetic samples, and ultimately in real samples involving blood samples from patients as well as additional healthy controls. The limit of detection (S/N = 3) was calculated as 44 pmol of target sequence in a 40-μl reaction volume in real samples.

1. Introduction

Janus Kinase 2 (Jak2) is a member of cytoplasmic non-receptor tyrosine kinases and is activated through members of the tyrosine receptor super family as growth hormone or erythropoietin receptors [1]. A Guanine-to-Thymine transversion, which results in a substitution of valine to phenylalanine amino acid at codon 617 of Jak2 (*JAK2V617F*), is responsible for the constitutive activity of this tyrosine kinase that activates signal transducer and activator of transcription (STAT), mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways, and transforms hematopoietic progenitor cells [2–5]. Presence of the *JAK2V617F* mutation is therefore a new diagnostic marker for myeloproliferative neoplasms (MPNs) as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). Detection of this mutation has also led to the development of selective Jak2 inhibitors that might be used in near future for the treatment of these hematological diseases [6–9].

JAK2V617F mutation can be detected in several different ways, each has specific advantages and limitations [10]. The most widely used techniques are allele-specific and quantitative Polymerase Chain Reaction (PCR) methods. In general, quantitative PCR methods are preferred due to monitoring disease progression [2,11]. Conventional DNA sequencing is another method of *JAK2V617F* mutation detection based on the Sanger DNA sequencing, which can distinguish a homozygous from a heterozygous genotype in the mixture [12]. Melting curve assay can detect the mutation by taking advantage of differences of the dissociation temperature of the primer either wild type (WT) or mutant type (MT) sequences. The first step of the melting curve analysis is the amplification of the region of interest, using PCR techniques such as real time PCR, in the presence of a double stranded DNA (dsDNA) binding dye that is expensive for routine lab operations. This method requires a fluorescence-tagged primer that is complementary to either WT or MT [13,14]. Additionally, one of the limitations of melting analysis is the possibility that another single point mutation interferes in the mutation of interest. This problem can be solved by using short amplicons in order to limit the probability of such an event. But this limits using of this method for each type mutations.

JAK2V617F mutation can also be analyzed by enzyme based restriction digest method. The G–T transversion destroys a *Bsa*XI enzyme restriction site and therefore results in a different restriction digest patterns. This approach is useful in discriminating homozygous from heterozygous genotypes [15].

Biosensor technology has comprised a real time, label free, sensitive and rapid approach to be used for DNA mutation detection as powerful

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alternative to above techniques. In recent years, there have been considerable progressions in the development of electrochemical DNA biosensors based on detection of the hybridization event between probe and its complementary sequence via intrinsic guanine oxidation signal [16], selective redox indicators and nanomaterials [17–19], resistance changes [20] and enzymatic tags [21]. Biosensors and biochips based DNA hybridization analysis has been widely used for the identification of specific DNA sequences in biomedical field and genetic researches particularly analysis of mutations, epigenetic changes and genomic sequencing. High sensitivity of such detection systems along with their compatibility with modern micro fabrication technologies prepares a basis for the future DNA microarray systems. Their portability, low-cost, easy applicable and minimal sample requirements make them excellent candidates for medical diagnosis, biomedical and biotechnological researches.

In this study, we demonstrated a label-free electrochemical DNA biosensor for the detection of single point mutation related to *JAK2V617F* using synthetic oligonucleotides and PCR amplicons as an alternative to agarose gel electrophoresis which is the classical method to identify the alterations of PCR samples after PCR. Due to the high importance of *JAK2V617F* mutation detection in various areas of biology and medicine, we attempted to develop a new biosensor system. Differential Pulse Voltammetry (DPV) technique was used to detect hybridization via intrinsic guanine oxidation signals changes. Detection of mutation in PCR samples was also characterized by Electrochemical Impedance Spectroscopy (EIS) with potassium ferri/ferrocyanide as a redox probe. In order to obtain an efficient discrimination between full complementary, single base mutation containing target (mismatch) and non-complementary target (NC), hybridization conditions were properly optimized. We mainly focused on achieving the successful discrimination by optimizing temperature of hybridization. The results of the study demonstrate a great promise for practical applications to detect *JAK2V617F* mutation in the development of clinical assay techniques. To our best knowledge, this is the first electrochemical detection method for this mutation in real samples by using a promising biosensor system. This biosensor system gives a hope for point of care testing in the future. The samples obtained from the healthy people and patients were analyzed with designed biosensor and found an alternative detection method to the above techniques for this mutation. Discrimination between WT and MT oligonucleotides and specificity of the designed sensor was achieved successfully for the first time with the electrochemical DNA biosensor.

2. Experimental

2.1. Apparatus and chemicals

DPV and EIS experiments were performed using the AUTOLAB PGSTAT-10-FRA (μAutolab type III, EcoChemie, The Netherlands) electrochemical analysis system. Boeco Thermo-Shaker for micro tubes was used for shaking and controlling temperature of the samples. The three electrode system consisted of the pencil graphite electrode (PGE) as the working electrode, a reference electrode (Ag/AgCl, Model RE-1 BAS, US) and a platinum wire as the auxiliary electrode. In brief, a mechanical pencil Model T 0.5 (Rotring, Germany), was used as a holder for pencil lead (Tombo, Japan), which were purchased from a local bookstore.

2.2. Synthetic oligonucleotides and real samples

Synthetic oligonucleotides (as lyophilized powder) were purchased from Alpha DNA (Canada) and used without further purification. Synthetic oligonucleotides sequences are:

Wild type probe (21-bp; WTP): 5′-GTC TCC ACA GAC ACA TAC TCC-3′
Wild type target (21-bp; WTT): 5′-GGA GTA TGT GTC TGT GGA GAC-3′

Single base mismatch (21-bp; MMT): 5′-GGA GTA TGT TTC TGT GGA GAC-3′

Non-complementary (27-bp; NC): 5′-TGG AGT ATT GAA GCT TTT GCC GAA GGT-3′

Stock solutions of oligonucleotides were prepared in three distilled water and stored at −20 °C. Diluted solutions of the oligonucleotides were prepared daily with 0.05 M phosphate buffer containing 500 mM NaCl pH: 7.4 (PBS). 0.5 M acetate buffer solution containing 20 mM NaCl pH: 4.8 (ACB) was used as a buffer of measurement.

JAK2V617F mutation in PCR product (163-bp):

5′AAGCAGCAAGTATGATGAGCAAGCTTTCTCACAAGCATTTGGTTTTAAATTAT**GGAGTATGTGTCTGTGGAGACG**AGAGTAAGTAAAACTACAGGCTTTCTAATGCCTTTCTCAGAGCATCTGTTTTTGTTTATATAGAAAATTCAGTTTCAGGATCACAGCT3′

In the PCR products, the bold parts indicate target sequences for *JAK2V617F* mutation and the underlined part shows the nucleotide where the mutation occurs. In the nucleotide sequences above, a Guanine (G) base changes to a Thymine (T) in affected people. NC sequences were selected from the HA gene of B/Kobe/1/94 (Gen Bank accession no: D38646.1).

2.3. Methods

2.3.1. DNA isolation and JAK2V617F mutation detection

Among the 61 human based samples, 13 are mutant and the rest is wild type. Genomic DNA was extracted from human peripheral blood leukocytes of the subject by using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) and stored at −20 °C until use. Genotyping of the subjects for the *JAK2V617F* mutation was performed by real-time PCR using the Light Cycler® v.2.0 instrument (Roche Applied Science, Mannheim, Germany). For this purpose specific primers and hybridization probes (TIB MOLBIOL, Berlin, Germany) for the analyzed mutation were used in combination with the Light Cycler DNA Master Hybridization Probes kit (Roche Applied Science, Mannheim, Germany). Alleles were identified by specific melting temperature (T_m) of the resulting amplicons after PCR and defined as 62 °C for the wild-type *JAK2* V allele (G nucleotide) and 54 °C for its mutant F allele (T nucleotide).

2.3.2. Electrochemical assay

The height of the guanine oxidation peak was used as analytical signal with DPV. Resistances of electrodes coated with oligonucleotides before and after hybridization were evaluated by EIS. The rapid detection procedure of the synthetic oligonucleotides and PCR amplicons consisted of the following steps: electrode pretreatment, hybridization, remove unbound bindings and measurement. Label free detection of *JAK2V617F* mutation by using synthetic oligonucleotides and PCR amplicons is illustrated in Fig. 1.

2.3.2.1. Electrode pretreatment. PGE was fixed vertically and immersed in solution of ACB and pretreated by applying +1.40 V for 30 s without stirring.

2.3.2.2. Hybridization.

a) *Hybridization with synthetic targets*: 5 μg/ml probe and 7 μg/ml target were mixed in PBS with 500 mM NaCl in a vial. These vials were stirred up for 20 min, at 68 °C and 600 rpm mixing speed. After gentle mixing, probe and hybrid solutions were put into the vials amounting to 40 μl each.
b) *Hybridization with real PCR samples*: The real samples obtained from the PCR amplification were diluted in PBS with 500 mM NaCl (dilution rate is 1:10); the diluted samples were then placed in a vial and denatured by heating at 95 °C for 8 min and applied ice shock for 1 min after heating. Then, desired concentration of probe solution

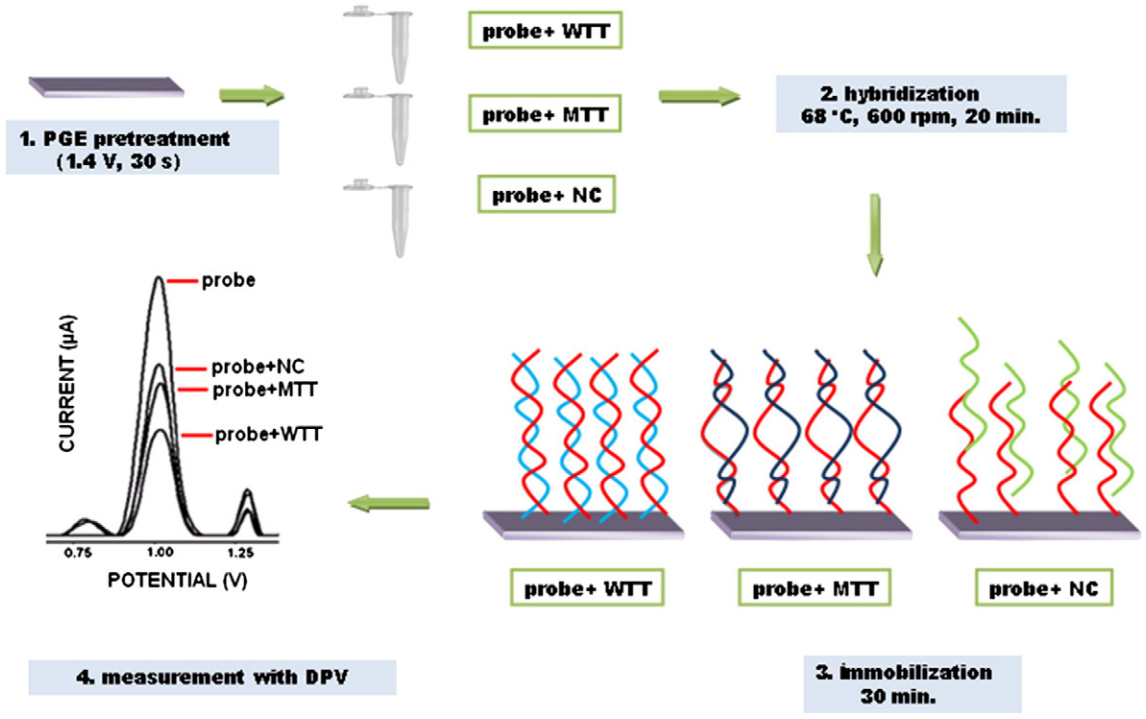

Fig. 1. Schematic presentation of proposed approach for electrochemical DNA biosensor for the detection of *JAK2V617F* mutation.

was added to the target. These vials were stirred up for 20 min at 68 °C and 600 rpm mixing speed. After gentle mixing, probe and hybrid solutions were put into the vials amounting to 40 μl each.

2.3.2.3. Immobilization. The pretreated electrodes were placed into these solutions and waited for 30 min. The electrodes were then rinsed with PBS with 500 mM NaCl for 1 min with stirring.

2.3.2.4. Electrochemical signal recording

2.3.2.4.1. Synthetic oligonucleotides. The electrochemical signals corresponding to guanine oxidation were measured by DPV from +0.75 to +1.4 V in ACB (modulation time = 0.05 s; interval time = 0.5 s; step potential = 10 mV; modulation amplitude = 50 mV). Repetitive measurements were carried out by repeating the above assay protocol on a new PGE surface. Each result is the mean and standard deviation of at least three measurements.

2.3.2.4.2. PCR samples. Hybridization was proved not only with DPV measurements but also with EIS measurements. EIS was performed with PBS in the presence of 0.5 mM Fe $[CN_6]^{3-/4-}$ at +0.24 V; a frequency range of 10 kHz to 50 mHz and an AC amplitude of 10 mV were applied. The impedance data were fitted to an equivalent circuit. The same protocol was done using a non-complementary sequence instead of the target sequence in order to control biosensor selectivity.

3. Results and discussion

In this study, an electrochemical DNA biosensor for the detection of *JAK2V617F* mutation by using synthetic and real samples was developed. First, we focused on achieving hybridization between probe and its complementary sequence. Then, our efforts turned to optimize conditions for the detection of any point mutation in the synthetic and PCR samples and also to maximize the selectivity.

3.1. Hybridization detection

The effect of experimental parameters including probe and target concentration, hybridization and accumulation time, ionic strength and hybridization temperature were explored for optimum analytical performance. As shown in Fig. 2A, the effect of probe concentration was studied by using synthetic oligonucleotides. In order to find optimum probe concentration, pretreated electrodes were dipped into vials containing from 1 to 15 μg/ml of probe concentration for 30 min. After probe immobilization, the electrode surface was rinsed once with PBS to remove unbound DNA. The saturation point was taken into consideration for optimum probe concentration. Five microgram per milliliter was chosen for further experiments.

In Fig. 2B, the effect of target concentration on hybridization was investigated. For target concentration study, concentration of probe was kept constant at 5 μg/ml and target concentration was increased from 5 to 15 μg/ml. The highest difference between probe and hybrid was observed at 7 μg/ml target concentration.

Next, the effect of hybridization time was analyzed. The influence of hybridization time is shown in Fig. 2C. Hybridization time was changed from 5 to 75 min. Five microgram per milliliter probe and 7 μg/ml target were mixed in PBS in a vial. These vials were stirred up from 5 to 75 min, at room temperature (25 °C) and 600 rpm mixing speed. The pretreated electrodes were put into these solutions and waited for 30 min. The electrodes were then rinsed once with PBS. The probe/hybrid (p/h) ratio increased from 5 to 75 min. There was no hybridization with 5 min and the p/h ratio increased after 5 min. Although the highest difference was observed at 75 min (decrease of guanine oxidation signal: 48.4%), 20 min was chosen as optimum hybridization time due to providing enough difference between probe and hybrid (decrease of guanine oxidation signal: 47.9%).

Different adsorption times were tried to find optimum DNA immobilization period. The immobilization of DNA to the surface of the electrodes by adsorption does not require any special reagents or any preferred nucleic acid modifications. An effective discrimination was obtained between probe and hybrid within 30 min adsorption time at room temperature. Also, the ionic strength is a critical factor for the stabilization of DNA duplexes. In order to optimize the ionic strength, the effect of the concentration of NaCl in the hybridization and rinsing buffers was carried out. Since DNA is a negatively charged biomolecule due to its phosphate backbone, the positive ions in the buffer solution

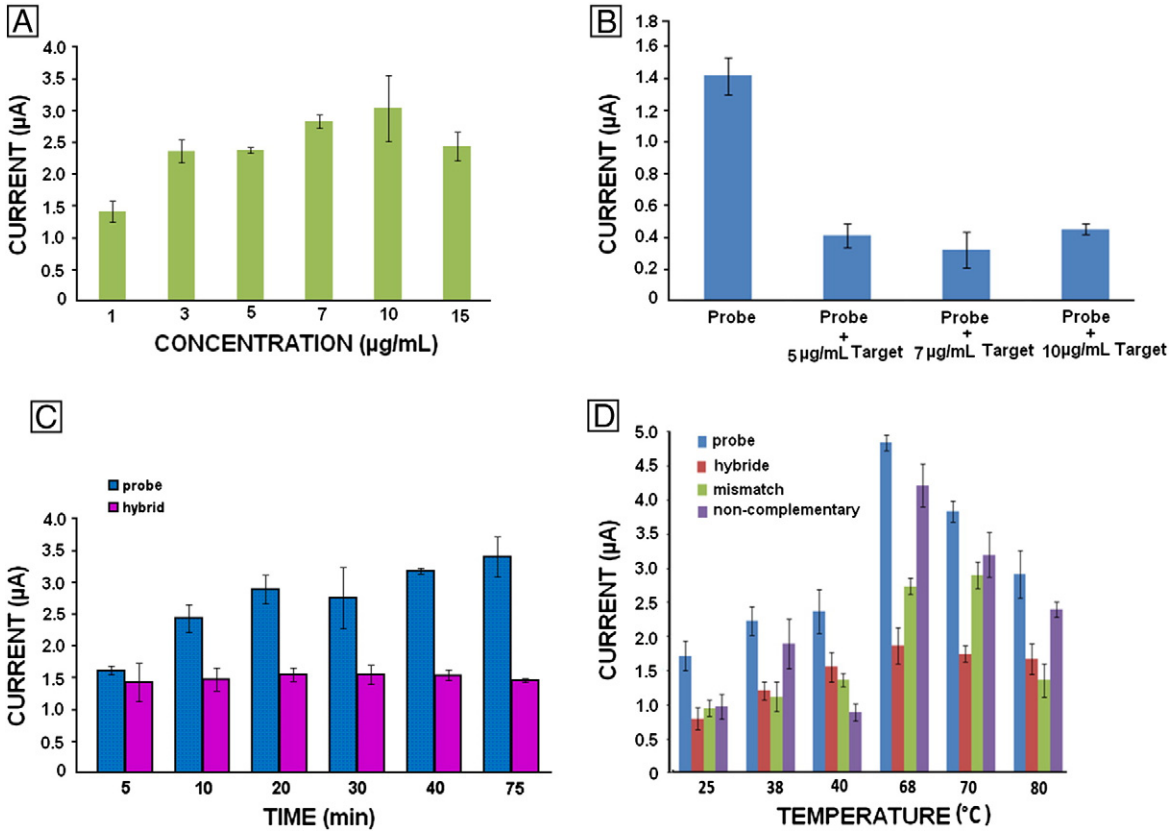

Fig. 2. Histograms based on guanine oxidation signals obtained from probe concentration (A), target concentration (B), hybridization time (C), and hybridization temperature (D) experiments.

neutralize the negative charges of the DNA, thus simplifying the hybridization of DNA strands. Therefore, NaCl concentrations of 20 mM, 100 mM, 200 mM and 500 mM and 1 M were tested. The strongest improvement of the hybridization signal was observed with 500 mM NaCl concentration in hybridization buffer (data not shown).

3.2. Selectivity study

Control experiments were performed to assess whether the biosensor responded selectively to the targets. Probe/complementary, probe/mismatch and probe/non-complementary couples were prepared to check the selectivity of biosensor related to *JAK2V617F* mutation at room temperature (25 °C). At above conditions, the decrease of the guanine signal was not enough to discriminate complementary and non-complementary sequences. So, we focused on hybridization temperature to overcome this problem due to hybridization temperature plays an important role in solution phase hybridization assay.

Melting temperature which could be defined as the temperature at half of the double strands are separated into single strands for the wild type, the mutant type and non-complementary target sequences were 67.7 °C, 66.3 °C and 77.1 °C respectively (Nearest Neighbor with 500 mM salt concentration). As it is known, at low temperatures and under optimum conditions, two complementary DNA strands usually form a double-helical duplex structure, which is stabilized by hydrogen bonds and by stacking interactions between base pairs. Also, when shorter DNA probes (<15-bp) are used, discrimination of single-base mismatch DNA can be readily achieved at room temperature. When longer probes (>15-bp) are used, control concentrations or hybridization time are not sufficient to destabilize unpaired duplex for single mismatch discrimination [22]. For this purpose, the effect of reaction temperature on the response of the designed sensor was investigated and is shown in Fig. 2D. Different hybridization temperatures were tried as 38 °C, 40 °C, 68 °C, 70 °C, 80 °C and compared to each other. Temperature was not increased more than 80 °C due to high temperature destabilizes DNA duplex.

Well defined peak differences between complementary and non-complementary targets were obtained with temperature of 68 °C. The interaction between probe and non-complementary oligonucleotides did not lead to a significant decrease of the guanine oxidation signal due to absence of hybridization. Hybridization at 68 °C for 20 min, the target was selectively bound to its complementary and became fixed on the biosensor surface. Discrimination between complementary and non-complementary sequences was achieved by controlling the hybridization temperature.

3.3. Mismatch discrimination

Providing differentiation between full complementary and a mismatch duplex is possible due to full complementary sequences are more stable than mismatch sequences. Moreover, hybridization is considered thermodynamically unstable when complementarities exist less than 6 nucleotides [23]. At 68 °C, mismatch containing synthetic oligonucleotide was also hybridized with probe and low guanine peaks were obtained as pseudo-hybridization occurred. Through the use of several washing steps, nonspecific adsorption effects were prevented. One time dipping, 5 s, 10 s, 15 s, 1 min and 5 min washing times with mixing were tried to take optimum result. The best result was obtained with 1 min washing time with mixing. The optimum conditions were applied to the experiment and the results are shown in Fig. 3A.

Fig. 3A-a shows oxidation signal of the only probe covered electrodes. A dramatic decrease in the peak current of guanine due to the hybridization reaction between the probe and complementary target sequences was obtained (b). The reason is that all guanines in the

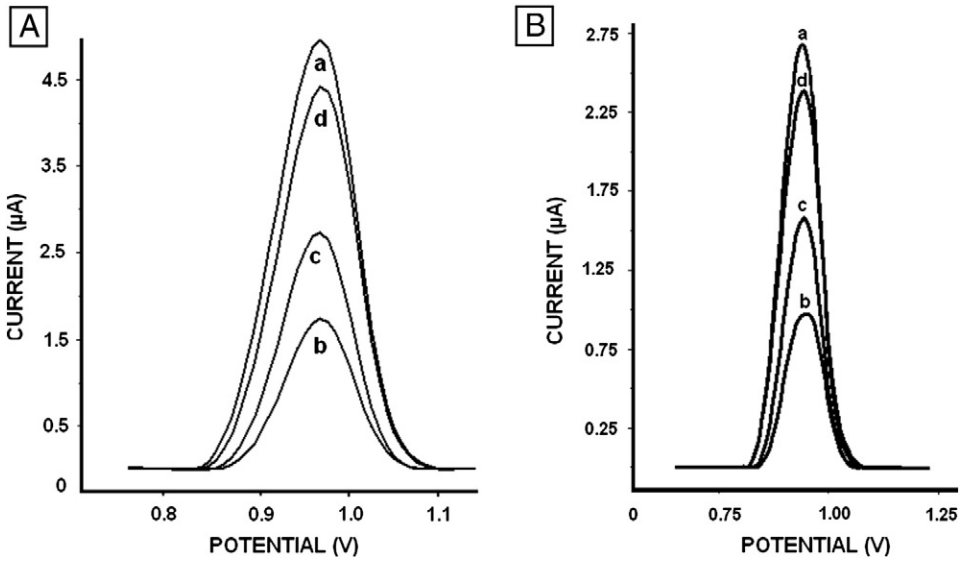

Fig. 3. Differential pulse voltammograms for the magnitude of guanine signal with synthetic oligonucleotides (A) and real samples (B). PGEs were modified with only wild type probe (a), wild type probe and complementary target (b), wild type probe and single mismatch target (c), and wild type probe and non-complementary target (d). The experimental conditions were as follows: PGE pretreatment: +1.4 V for 30 s without stirring; hybridization: 5 μg/ml probe and 7 μg/ml complementary target for synthetic target, 1:10 denatured PCR amplicon for real samples at 68 °C temperature and 600 rpm speed for 20 min; adsorption for 30 min; rinsing electrodes for 1 min with mixing; and DPV measurement by scanning between +0.75 and +1.4 V in ACB. The curve of I (μA) vs. E (V).

probe DNA were partly closed to oxidation after hybridization. Mismatch sequence has only one base different from the complementary target, but a noticeable difference was provided successfully (c). This data demonstrates that only complementary target could form an entirely matched hybrid structure with the probe, resulting in a significant decrease on the magnitude of the guanine signal. In the presence of non-complementary, higher guanine signal than complementary was observed (d). This clear difference between full match and non-complementary indicated that hybridization did not occur with a non-complementary sequence.

A series of 3 repetitive measurements of the oxidation of guanine resulted in reproducible results with a relative standard deviation (RSD) of 2.26%, 8.83%, 4.37% and 11.8% for synthetic probe (a) hybrid (b), mismatch (c) and non-complementary (d) oligonucleotides respectively.

3.4. Real sample analysis

In the biosensor systems, it is very important to test the method with PCR products obtained from clinical samples. In Fig. 3B, voltammograms show the guanine oxidation signals obtained with real PCR samples. As it is shown in Fig. 3B, well defined peaks were observed with probe (a), hybrid (b), mismatch (c) and non-complementary (d) sequences. The highest guanine signals were obtained from the synthetic probe immobilized electrode (a). As a result of the hybridization with denatured PCR sample, the magnitude of the guanine peak decreased to approximately half of the original value (b). When the probe and mismatch mixed, the decrease was higher than the hybrid (c) as a proof of specificity of the designed sensor. Since the mismatched duplex is not as stable as complementary, differentiation between both them can be monitored. After interaction between probe and non-complementary oligonucleotides, a slightly higher signal than the hybrid was obtained (d).

A series of 3 repetitive measurements of the oxidation of guanine resulted in reproducible results with a relative standard deviation (RSD) of 4.32%, 6.37%, 5.26% and 8.01% for synthetic probe (a) hybrid (b), mismatch (c) and non-complementary (d) oligonucleotides respectively. The detection limit was calculated as 44 pmol in 40 μl reaction volume. With this biosensor system, we were able to detect the presence of a low level of *JAK2V617F* in a small fraction of mutation contained blood samples.

These PCR amplicons were long and consisted of 163 base pairs while synthetic targets have 22-bp. Although, guanine based method allowed us to detect this mutation, DPV measurements could be supported by another method. These observations were further confirmed by EIS measurements. Due to PCR samples have lots of free guanines, the sequences that are not connected with the probe could have been oxidized. Actually, in one study related to oligonucleotide length effect, Kalantari et al. used targets of 38, 44, 107, 1788 and 2907 base pairs coupled with their probes of 16 and 22 base pairs. Their results declared that target length had only a small effect on the normalized signals [24].

According to these findings, we tried to detect the hybridization between probe and PCR amplicons with EIS. The basic principal of impedance measurement is to evaluate the resistance changes between probe and hybrid/mismatch/non-complementary sequences. Impedance spectroscopy exposes an increase of the electron transfer resistance when the hybridization occurs since an increase of negative charge density associated with phosphate groups of DNA [25] which allows us to screen the hybridization event. Fig. 4 shows the electrochemical impedance spectra of PCR amplicons before and after hybridization (Nyquist plots, Z′ versus Z″). Each point represents a value of Z′ and Z″ measured at 0.24 V ac frequency. The semicircle describes the Faradic

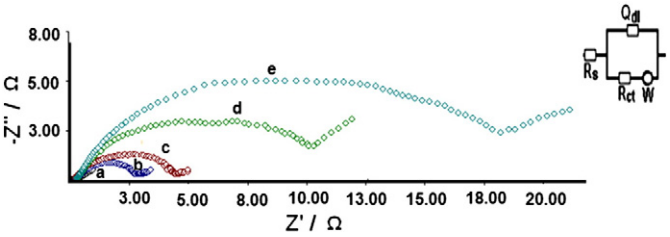

Fig. 4. Nyquist diagrams (imaginary part Z″ vs. real part Z′) in PBS in the presence of Fe $[CN_6]^{3-/4-}$ recorded after each modification step: at bare PGE (a), probe (b), non-complementary (c), mismatch (d) and hybrid (e) coated electrodes. Other elements in the equivalent circuit were as followed: Q_{dl}, constant phase element related to double-layer capacitance; R_{ct}, charge-transfer resistance; W, Warburg impedance; R_s, solution resistance.

electron-transfer process at the electrode surface, and the linear portion contains information about the diffusion-limited transport of the redox ions near the electrode.

In order to determine hybridization, mismatch discrimination and specificity of the sensor, charge transfer resistances (R_{ct}) were measured. The charge-transfer resistance, R_{ct}, is the key feature that allows us to discriminate matched DNA from mismatch DNA. Due to having negative phosphate backbones, DNA immobilization onto the electrode surface creates a negatively charged interface. In the experiment, Fe $[CN_6]^{3-/4-}$ was used an anionic redox marker, and it was pushed by DNA from the surface. This interaction cause increased resistance value when it is compared with bare electrodes and DNA covered electrodes. The bare electrode (a) was covered with probe (b). After covering the electrodes with probe, these electrodes were interacted with full match target (e), mismatch target (d) and non-complementary target (c). The interfacial electron transfer resistance value increased maximum after hybridization (e) due to full duplex formation and steric hindrance. The resistance signals obtained from mismatch (d) and non-complementary (c) were lower than that obtained from the hybrid (e), which indicates specific hybridization. According to the results, it was evident that the developed strategy had offered a specific platform for distinguish effectively mutant target DNA and wild target DNA.

By evaluating the impedance spectroscopy measurements, it is possible to determine DNA surface coverage (θ) of the graphite electrode surface, according to the following equation [26] where R_{ct}^{bare} (Ω) and R_{ct} (Ω) are the charge transfer resistances before and after electrode modification with probe and hybrid:

$$(\theta) = 1 - \left(R_{ct}^{bare}/R_{ct}\right).$$

From the impedance spectra displayed in Fig. 4, charge transfer resistances of bare graphite electrode, probe and hybrid covered electrodes are 155, 1980 and 9400 Ω, respectively. The corresponding surface coverage values of the probe and hybrid modifications were calculated as 92% and 98% respectively.

4. Conclusion

The designed biosensor is able to detect the complementary sequence in real samples and also exhibited high sensitivity and discrimination ability even for the detection of single-base mismatch. A biosensor with such a low detection limit (picomole level) is suitable for clinical operations. With this highly sensitive and specific diagnostic method, it is simple to achieve a large quantity of clinical samples in laboratories within an hour. Our analysis costs approximately less than $1 per sample (1 box pencil graphite supply us 24 measurements). Compared with the traditional mutation screening methods, detection of *JAK2V617F* mutation is simple and nontoxic; thus, it is a promising method for the potential use in medical diagnosis of the *JAK2V617F* mutation. Additionally, it is an alternative to agarose gel electrophoresis which is labor intensive, redundant, and involves the use of ethidium bromide and ultraviolet light. Our future research will focus on to design high-throughput clinical testing devices based on microarray and lab-on chip technology and also produce suitable kits.

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