[Frontiers in Bioscience 10, 2518-2526, September 1, 2005]

IMPEDANCE LABELLESS DETECTION-BASED POLYPYRROLE PROTEIN BIOSENSOR

C. M. Li [1], Wei Chen [1], Xu Yang [1,2], C. Q. Sun [3], Crystal Gao [4], Z. X. Zheng [4] and Jaymie Sawyer [4]

[1] School of Chemical & Biomedical Engineering, Nanyang Technological University, Nanyang Avenue, Singapore 639798. [2]College of Life Science, Central China Normal University, Wuhan 430079, China. [3] School of Electrical and Electronic Engineering, Nanyang Technological University, Nanyang Avenue, Singapore 639798. [4] Motorola Life Science, Tempe, Arizona 85287, USA.

TABLE OF CONTENTS

1. ABSTRACT

A simple and sensitive electrochemical immunosensor with impedance labelless detection and novel data processing method was investigated. One-step polymerization was used to electrochemically deposit an antibody impregnated polypyrrole film on a glassy carbon electrode surface for the immunosensor. Impedance measurements provided a labelless or reporterless method to detect antibody (Ab)-antigen (Ag) interactions. Dimensionless analysis was employed to successfully process the measured impedance data. Since the method derived unit impedance change to eliminate or reduce the variation of the bulk electronic properties of Ab/polypyrrole films, the signal to noise ratio (S/N) was significantly improved for high sensitivity and specificity. Nonspecific binding effect was studied by array electrode chips and was found out that the polypyrrole electrode without antibody attachment had much stronger nonspecific binding effect than the Ab/polypyrrole electrode; incubation followed by thoroughly washing significantly reduced the nonspecific interference. 10 pg/ml detection limit and superior specificity were achieved by the method, demonstrating a highly sensitive labelless immunosensor in comparison with the detection limit of ng -μg/ml for the reported polypyrrole based immunosensors. The electrochemical immunosensors presented in this paper, due to its simplicity, low cost, high sensitivity and superior specificity, could be an invaluable tool for clinical diagnostics and could have potential applications in drug discovery, environmental and food analysis.

2. INTRODUCTION

Immunoassay has become a powerful analytical technique since 1970s due to its capability of direct, sensitive and specific detection in diagnostic applications (1). Accordingly, electrochemical immunoassay has been grown greatly and made significant advances (2-9) because its measurements are simpler, less expensive, and even more precise over optical detection methods.

Conductive polymers such as polyaniline, polythiophene, polypyrrole in electrochemical sensors have been extensively investigated (10-23). Polypyrrole is one of the conductive polymers mostly used in electrochemical immunosensors (24-29) because of its superior biocompatibility, simple synthesis, and easy immobilization of various biological compounds. Polypyrrole thin films can be electrochemically deposited on different electrode substrates by cyclovoltammetric, galvanostatic and potentiostatic methods (13, 28, 30-34). Electrochemical detection of immunointeraction can be conducted both with and without labeling. A frequently used detection scheme is based on amperometric methods, where proteins are labeled with enzymes to convert the substrate into an electroactive product (35). Direct labelless detection measures changes in capacitance and/or resistance induced by Ab-Ag bindings on the electrode. Conductivity could simply provide a measure of the ionic concentration and mobility in a solution. However, the measurements are difficult due to the variable ionic background of clinical samples and the relatively small conductivity changes observed in high ionic strength solutions (36). Potentiometric method can offer a labelless detection, in which the surface potential changes at an ion-selective electrode are used to detect the ionic product of an enzymatic immunoassay reaction. Typically, potetiometry is less sensitive and more slowly responsive than amperometry, and is limited by fewer enzymatic reactions that can be employed by the method (37). Capacitive measurements have been used for the real-time and labelless detection of Ab-Ag interactions. Generally, the method is limited to detect relatively large molecules, and requires more precision of capacitance measurements with commercial instruments (38). Impedance labelless detection for DNA hybridization has been reported by Li (39, 40) and Lee (41). Li and coworkers further reported the impedance labelless detection of protein bindings in a US patent (42). One challenge for a labeling method in immunoassays is the requirement of the second antibody for labeling. In some cases, the labeling method cannot be used when the target protein molecule is too small to have an additional epitope for the second antibody binding. Besides, the labeling method is very time-consuming, labor-intensive, and expensive. Thus, labelless or reporterless electrochemical immunosensors become extremely attractive. The polypyrrole immunosensors including the labelless methods reported so far were not sensitive and demonstrated detection limits from ng/ml to μg/ml range (11, 12). There remains a need to develop less expensive, more sensitive and reproducible polypyrrole based immunosensor, particularly the labelless immunosensors for practical medical diagnostic applications.

In this paper, we reported the advantages of a new impedance labelless detection approach to monitor the Ab-Ag bindings. The method employed simple electrode preparation and data analysis method to achieve high sensitivity and good specificity. The method demonstrates great potential applications in clinical diagnostics, drug discovery, environmental control and food analysis.

3. METERIAL AND METHODS

3.1. Materials and solutions

Pyrrole, Leptin, Anti-axidothymidine (AZT) and streptavidin (SA) were purchased from Aldrich. The received pyrrole was distilled and kept in refrigerator prior to use. Unconjugated Rabbit anti-BAP (Bacterial Alkaline Phosphatase) and rabbit anti-gp120 (unconjugated), the outer envelope protein of HIV-I, were purchased from Biodesign and Virostat, respectively. Fetal bovine serum (FBS) was purchased from Fisher, USA. Different grades of Gamma alumina powder were purchased from CH Instruments, Inc, USA. The deionized water used in all experiments was produced by a Millipore milli-Q water purification system. All pipette tips were sterilized by autoclaving for 2 hours. All other chemicals were of analytical grades and obtained from common commercial supplies.

3.2. Apparatus

EG&G PAR 273 potentiostat was used to conduct cyclic voltammetry for activation of the glassy carbon electrodes and electrochemical deposition of the Ab/polypyrrole film on the electrode surface. AC impedance measurements were carried out with 1260 Impedance Gain-Phase Analyser/1287 Electrochemical Interface system (Solartron Inc., Houston, Tex.). A Ag/AgCl, saturated KCl reference electrode was used. A coiled platinum wire was used as the counter electrode. All experiments were conducted at room temperature.

Glassy carbon electrodes (3 mm in diameter) used in all experiments were purchased from CH Instruments, USA. Before use, the surface of the glassy carbon electrode was polished with 0.3 micrometers gamma alumina powder following by rinsing with deionized water, and then polished with 0.005 micrometer powder. After polishing, the electrodes were ultrasonically cleaned for 5 min. in deionized water, and then soaked in acetone for 5 min. following vigorously washing with deionized water. Finally, immersed the electrodes in 0.5 M sulfuric acid for 5 min., and again washed vigorously with deionized water.

Silicon array chips, shown in Fig.1a, used in all experiments were made by the Process Lab of Motorola Research Center, USA. The chip was constructed on an oxidized silicon substrate with a well structure where 4 individual 100x100 μm^2 gold working electrodes located in the bottom of 4 wells. The counter electrode is a square strip gold electrode on the top side symmetrically surrounding the 4 well working electrodes (Figure 1b). The surface area of the top electrode is larger than the bottom array electrodes by 1 order of magnitude. In ac impedance

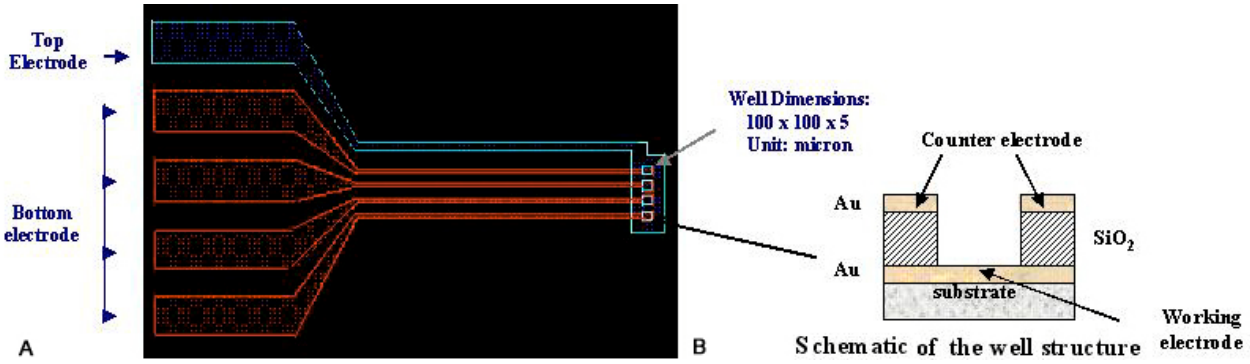

Figure 1. Silicon array chip. 1a: Silicon chip 1b: Schematic of the electrochemical cell structure.

measurements, the bottom array and the large electrodes served as working electrodes and the counter electrode, respectively. Since the counter electrode has much lower impedance than the working array electrode due to its larger surface area, its impedance contribution could be neglected in analysis of measured impedance data. Before use, the array chip was cleaned with the same as the procedure used for the glassy carbon electrodes, but without polishing.

3.3. Experimental procedure

For immobilization of probe proteins into polypyrrole film matrix, simple one-step electrochemical polymerization in PBS (phosphate buffer solution) containing 0.05 M pyrrole and a probe protein, an antibody in most cases, with a concentration of 150 μg/ml was conducted. Before electrochemical polymerization, the glassy carbon electrodes were activated in a PBS by scanning from 0 to 1.8 V at 100mV/s and then holding at 1.8 V for 60 seconds. The cleaned electrodes were kept in a PBS until deposition. A 0.2 mA/cm^2 constant current was applied on the electrode for 100 seconds to electrochemically produce the Ab-entrapped polypyrrole film.

The AC impedance baseline of the polypyrrole immunosensor prepared was first measure in a PBS in the absence of target antigens. The electrodes were then immersed in a target antigen solution in a sealed vial for 1 hour incubation. The sealed vial was used to eliminate electrolyte evaporation for reducing change of analyte concentration, which was particularly important for detections of analytes with low concentrations. After the incubation, the resultant electrodes were thoroughly rinsed at room temperature with copious PBS and then AC impedance was measured again in a PBS. All AC impedance measurements were recorded at open circuit voltage (OCV) vs. Ag/AgCl in a PBS in frequency range over 1Hz-1MHz except additional notice.

4. RESULT AND DISCUSSION

4.1. Impedance detection of Ab-Ag interactions

1 ml of 1.5 mg/ml Virostat anti-AZT was added to 10 ml of 0.05 M pyrrole/PBS to prepare the polymerization precursor solution, in which the polymerization was applied to deposit anti-AZT/polypyrrole films on glassy carbon electrodes. The resultant electrodes were used in AC impedance measurements in a PBS before and after incubation in a 100 ng/ml AZT/PBS solution. The measured complex impedance (Z) versus frequency, known as Nyquist Plot, from the prepared polypyrrole/AZT glassy carbon electrodes is shown in Figure 2. Impedance measurements with a frequency response analyzer such as Solartron 1260 used in our experiments could provide more information for qualitative and quantitative analysis than methods with conventional conductivity and capacitance measurements at a fixed frequency. Ideally, Nyquist Plot has a well-defined frequency-dependent semicircle curve at the high frequency range following by a straight line. The complex impedance is the sum of Z', the real components mainly from resistance, and Z", the imaginary components from capacitance, inductance and other distribution components. Randle equivalent circuit (42) is frequently used to model the complex impedance in an electrochemical cell, which is composed of the ohmic resistance of the electrolyte solution, R_s in connection in series with parallel elements of double layer capacitance, C_{dl}, and Faraday impedance, Z_f. The parallel elements C_{dl} and Z_f versus frequencies form a semicircle. Z_f often comprises serially connected electron-transfer resistance, R_{et}, and Warburg impedance, Z_w, a resistance from diffusion of ions from the bulk electrolyte to the electrode surface. The impedance measured at the anti-AZT/polypyrrole electrode after incubation in AZT/PBS (curve 2 in Figure 2) shows significant change than that before the Ab-Ag interaction (curve 1 in Figure 2). The diameter of the semicircle apparently increased after Ab-Ag interaction, indicating R_{et}, the electrochemical reaction resistance increased.

It has been reported that the increase of R_{et} was due to the hindrance of the hybridized DNA to the anionic doping process of polypyrrole, which was used for labelless detection-based DNA sensors (13). The larger the diameter of the semicircle, the higher the R_{ct}. The hindrance to the doping/undoping process was caused most possibly from steric barriers, the hybridized target DNAs. Similarly, the bound protein molecules in the Ab/polypyrrole matrix could create the same steric obstruction to the ion doping

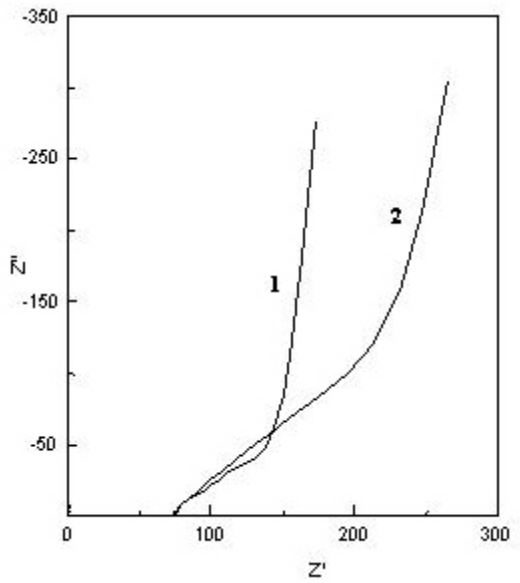

Figure 2. Impedance complexes at the anti-AZT/polypyrrole film/glassy carbon electrode before and after incubation for 1 hr in a PBS solution containing 100 ng/ml of AZT. Curve 1: before incubation; curve 2: after incubation.

process. Thus, R_{et} increase after incubation shown in Fig.2 indicated that the doping/undoping process in the Ab/polypyrrole film also was slowed down by the Ab-Ag interaction. The change of the impedance could be used to detect the Ab-Ag binding. However, the results reported in literature (13) showed better defined semi-circles at DNA/polypyrrole biosensors than that in Fig.2. This indicated that the Ab-Ag binding in the conductive polymer has stronger effect on the doping/undoping process. However, it is difficult to analyze the impedance results with Randle equivalent circuit for R_{et} and other related electronic parameters, since a modeling on a distorted semicircle could introduce great deviations from the true value. To solve this problem, Willner et al and Kanungo introduced additional reporter, $Fe(CN)_6^{-4/-3}$ in their impedance measurements (12, 20, 39) to detect IgG (12),liposomes/bioton labeled target antibody and DNA (20, 39) for the enhanced amplification of Ab-Ag binding sensing processes. The mechanism is clear since $Fe(CN)_6^{-4/-3}$ was used as reporters to probe the precipitation of labeled large molecules on electrodes, which could retard the electron transfer between the $Fe(CN)_6^{-4/-3}$ redox pair to increase R_{et} after Ab-Ag binding, which are not labelless or reporterless Apparently, results obtained with labelless or reporterless impedance detection should have new date analysis method, which will be discussed later.

4.2. Data processing for S/N(signal to noise) ratio improvement

AC impedances were measured using the anti-AZT/polypyrrole electrodes before and after incubations in PBS containing 0, 10, 100, 1000, 10,000, 100,000 and 1000,000 pg/ml of AZT, respectively. PBS without AZT composition (0 pg/ml) was used for the negative control experiment. In all experiments, three electrodes were used in measurements for every concentration to determine standard deviation. Typical Nyquist Plots of the impedance measurements for 0, 10, 100, and 1000 pg/ml of AZT are shown in Fig.3(a) and the impedance results obtained with two electrodes in the solutions containing 0 and 100 ng/ml AZT are illustrated in Fig.3(b). In Figure 3, the curves 1,3 and 2,4 represent the impedances before and after incubation, respectively; while the curves 1,2 and 3,4 display the impedances in the solutions containing 100 and 0 ng/ml AZT, respectively. It is hard to differentiate the varied low concentrations based on the impedances in Fig.3(a). Furthermore, the two electrodes in Fig.3(b) are supposed to have the same impedance before Ab-Ag interaction, but it shows great variation in impedances. Deviations of apparent surface area of electrodes from each other could result in impedance variation. However, the resultant errors should be less than 4% in terms of the measured deviation of the electrodes, which is much smaller than the results in Fig.3 (b) where instability of the Ab/polypyrrole film was also exhibited because impedance change was observed before and after the incubation in 0 AZT solution. This may be attributed to the variation of the deposited polypyrrole film features since electrochemical deposition process is greatly influenced by the chemical and physical properties of the electrode surface. It was unlikely occurred for different electrodes to have identical deposited polypyrrole films with the same surface properties even the electrode treatment and deposition condition was well-controlled in the experiments. It is not practical to use simple subtraction of impedance changes before and after the incubation for a relationship between impedance responses and concentrations of antigen when a number of electrodes are employed in the labelless impedance detection for Ab-Ag interactions. Actually, the simple subtraction method leads a messy plot between impedances and concentrations of AZT, which shows a scattering pattern of figure.

In order to eliminate or reduce the variations from different single electrodes in multi-concentration analysis, a concept of normalized dimensionless impedance unit change was introduced to analyze the measured impedance data. In this method, for example, the resistances measured at an Ab impregnated electrode before and after the target antigen incubation are assumed as R_1 and R_2, respectively. The normalized resistance unit change, ΔR_N, is

$$\Delta R_N = \frac{R_2 - R_1}{R_1} \qquad (1)$$

The physical meaning of ΔR_N is the dimensionless unit resistance change. This normalization is different from the normalized change of intensity used in array biochips, which uses the responses at biomarked spots to be divided by the responses at the negative spots, and also is different from the normalized resistance change ($\Delta g/g_o$) used in literature (12) where $\Delta g = g - g_o$; g_o is the conductance of the sensor without any interest analyte and g is the conductance of the same sensor in presence of the analyte. The normalized dimensionless unit resistance change introduced here is based on results from a single electrode.

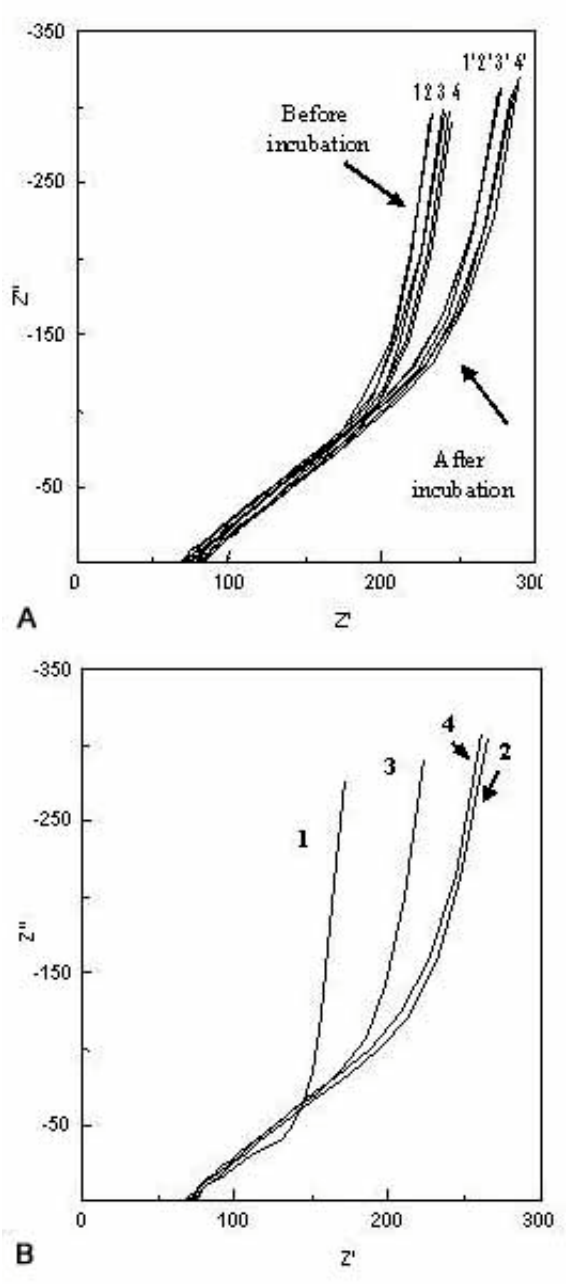

Figure 3. Impedance results using anti-AZT/polypyrrole electrodes before and after incubation in (a) AZT/PBS solutions containing 0, 10, 100, and 1000 pg/ml antigen AZT where curve 1,2,3,4 and 1',2',3', 4' represent different sensors used to detect 0, 10, 100, and 1000 pg/ml AZT, respectively before and after incubation. (b) in AZT/PBS solutions containing 0 and 100 ng/ml antigen AZT where curve 1, 2 and 3, 4 measured before and after the incubation in 100 and 0 (negative control) ng/ml, respectively, and 1, 3 and 2, 4 measured before and after the 1 hour incubation, respectively.

In most cases, a single immunosensor cannot be used for multi-concentration analysis. When multi electrodes are used for multiconcentration analysis, the dimensionless unit impedance change could mainly quantify the change resulting from the Ab-Ag interactions in the polymer matrix, rather than the change of the bulk electric properties of polypyrrole films for eliminating or reducing the variation of bulk resistances caused by different Ab/polypyrrole films. Furthermore, since there were no well-defined semicircles in the measured impedance for Randle model, it was essential to analyze R_{et} with other approaches. During data analysis, it was found out that a frequency or a band of frequencies for measured impedance had the best linear relationship between the resistance and the concentration of Ag. The frequency or frequency band was different for different Ab-Ag bindings, which was from few Hz to hundreds Hz. The most significant part of the impedance at the frequency is equal to R_{et} since R_{et} is proportional to the concentrations of Ag. A software was designed to directly analyze impedance data for R_{et} at different frequencies, and then to find out the frequency or frequency band to obtain the best linear relationship between ΔR_N and concentration of Ag. Using this dimensionless analysis method, the impedance results measured described above (a part of typical curves shown in Figure 3) were processed and was found out that the frequency band for the R_{et} was around 50 – 60 Hz. Figure 4 is the processed results to show a plot of ΔR_N vs. different AZT concentrations for the measured impedance results at different electrodes, in which the ΔR_N linearly increases with AZT concentration increase over 4 orders of magnitude, and then reaches the saturation plateau. The results exhibit satisfactory standard deviation, an excellent detection limit of 10 pg/ml, and a typical Ab-Ag binding characteristic behavior. The detection limit is at the similar level to those of the best immunoassay methods that have been reported (43). Different concentrations of BAP, gp120, lepton, SA and biotin were detected with this labelless impedance method, and linear dose responses to all these antigens with detection limit of 10 pg/ml were achieved.

AZT is a low molecular weight analyte, being too small to have multiple epitopes, and its analysis is not amenable to sandwich type assays wherein the analyte has to be bound both on the first antibody (identifier for analyte) and on the second labeled antibody to allow detection. The highly sensitive labelless detection technique for small molecule like AZT is particularly significant because its significant applications in the interaction between antibody and a small molecule such as drug organic molecules, toxins, etc.

4.3. Specificity of impedance labelless detection of proteins

Anti-AZT, anti-SA, anti-lepton, anti-BAP and anti-gp120 were used to prepare different Ab/polypyrrole film electrodes for immunosensors using the method described in 3.3. Each of these Ab/polypyrrole immunosensors was placed in different PBS solutions containing 25 ng/ml of different antigens to validate the specificity of the labelless impedance immunosensors. The experimental results in Figure 5 show the crossover reaction rates of each Ab/polypyrrole immunosensor against different antigens.

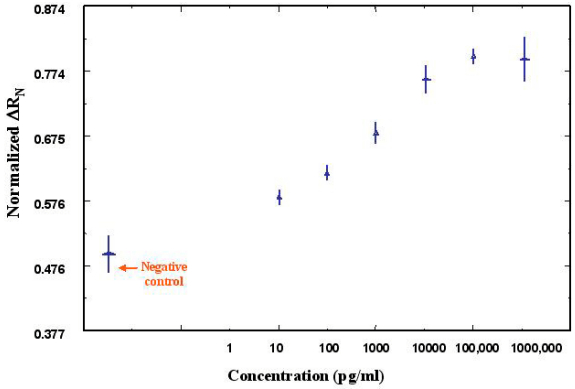

Figure 4. Relationship of Normalized Dimensionless Resistance Change, ΔR_N vs. AZT concentration.

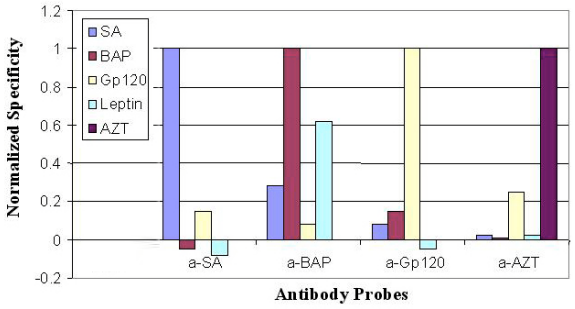

Figure 5. Specificity of different Ab-polypyrrole protein sensors. Concentration of all tested target antigens was 25 ng/ml in PBS.

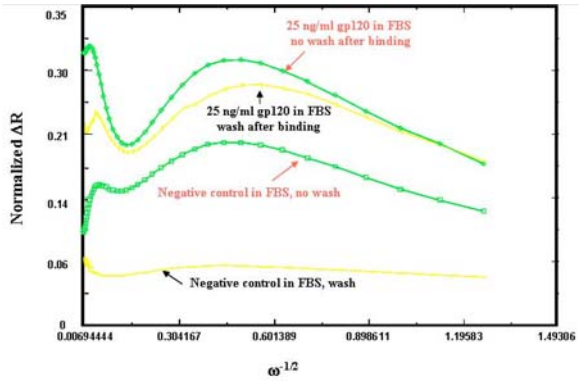

Figure 6. Nonspecific binding effect on Single to noise ratio for both anti-gp120/polypyrrole film and plain polypyrrole film electrodes.

Anti-AZT, anti-SA, anti-lepton, and anti-gp120 based on polypyrrole immunosensors demonstrate good specificity against other antigens. However, the anti-BAP/polypyrrole sensor had 60% crossover rate with lepton in comparison with its paired BAP Ab-Ag binding. We used ELISA method to determine the crossover reaction rate between anti-BAP and lepton. It was found out ~61% crossover reaction rate of anti-BAP against lepton. The crossover reaction rate measured from the labelless electrochemical immunosensor was very well in agreement with the measured results by ELISA. ELISA has been the mainstay of testing in all fields of pure and applied biology, and in particular, now constitutes a backbone diagnostic technique (44). Thus, the crossover reaction rate of anti-BAP with lepton occurred at the BAP/polypyrrole immunosensor was not due to failure of the impedance labelless detection technique.

4.4. Nonspecific binding effect in serum sample

The silicon array chip contains four gold microelectrodes located in the bottom of the four wells in the chip. The polypyrrole film without anti-body was deposited on the two gold electrodes as negative controls. The other two gold microelectrodes were employed for anti-gp120/polypyrrole film deposition. The prepared bioarray chip was immersed in FBS containing 25 ng/ml gp120 for Ab-Ag interaction following by immediate ac impedance measurements for the four array electrodes as the baselines. After incubation for 1 hour in a PBS, ac impedance measurements were conducted again at the bioarray chip directly in the serum. Then, the bioarray chip was taken out and washed thoroughly with PBS. Impedance measurements were carried out in a PBS without any antigen. Plots of ΔR_N (average value for two electrode data) vs. frequency obtained from experimental results are shown in Figure 6, where the curves have indication of their measurement conditions. Reasonable difference between the positive response and negative control is observed for the direct impedance measured in the serum. However, after washing ΔR_Ns from both the negative control and the anti-gp120/polypyrrole electrodes were reduced. The reduction of ΔR_Ns with PBS washing indicated there was nonspecific bindings occurred during incubation in serum. However, the signal reduction of the negative controls with PBS washing was much more significantly than that of the positive responses, indicating that the nonspecific binding on the polypyrrole electrodes was much stronger than that of anti-gp120/polypyrrole electrodes. This suggests that wash is a necessary step to significantly improve the measurement S/N ratio to enhance high sensitivity and specificity.

5. CONCLUSION AND PERSPECTIVES

A simple and sensitive impedance labelless detection based on electrochemical immunosensor and its data processing method was investigated. One-step polymerization was used to impregnate antibody into the polypyrrole film on glassy carbon electrodes for producing electrochemical immunosensors. The experimental results showed that the labelless impedance detection method could be used to detect different antibody-antigen interactions. Normalized dimensionless unit for impedance change was introduced to successfully analyze the measured impedance data. The derived method using resistance change is exploited to eliminate or reduce the variation of the electronic properties of Ab/polypyrrole films, leading S/N ratio significant improvement for high sensitivity and specificity. The concept of normalized dimensionless unit change could be used in existing bioarray chips and other sensors for improvement of S/N ratio. 10 pg/ml detection limit and superior specificity were

demonstrated by the methods. The reported immunosensor, which allows the quantitative analysis for unlabeled analytes, could be an invaluable tool for clinical diagnostics and could have potential applications in drug discovery, environmental and food analysis due to its simplicity, high sensitivity and low cost. With many therapeutic drugs like AZT there is a fine line between efficacy and toxicity, and rates of drug metabolism differ among people and states of disease for an individual patient. Based on this labelless impedance detection technology, a biochip could be developed with different antibody-attached probes to detect different antigens for high throughput analysis. In addition, PBS wash demonstrated the improvement of S/N ratio by reducing nonspecific protein bindings on the electrodes. The developed immunosensor needs to be further tested in patient samples and block agents could be next research area for further reducing the nonspecific bindings. The sensitivity improvement could be possibly further accomplished by overcoming the steric and kinetic barriers of entrapped probe biomolecules from the surrounding polypyrrole molecules. High efficient covalent method could be the solution, which has been currently studying in the author's lab.

6. ACKNOWLEDGEMENT

The authors are grateful to Motorola Process Lab of Motorola Research Center in Tempe, Phoenix for fabricating silicon based array chips for the experiments.

7. REFERENCES

1. Ronkainen-Matsuno, N.J, J.H. Thomas, H.B. Halsall & W.R. Heineman: Electrochemical immunoassay moving into the fast lane. *Trends Anal Chem* 21, 213-225 (2002)

2. Thomas, J.H, S.K. Kim, P.J. Hesketh, H.B. Halsall & W.R. Heinemana: Microbead-based electrochemical immunoassay with interdigitated array electrodes. *Anal Biochem* 328, 113–122 (2004)

3. Lei, C.X, J. Wu, H. Wang, G.L Shen & R.Y. Yu.: A new electrochemical immunoassay strategy for detection of transferrin based on electrostatic interaction of natural polymers. *Talanta* 63, 469–474 (2004)

4. Kuramitz, H., Matsuda M., Thomas J.H., Sugawara K. & Tanaka S: Electrochemical immunoassay at a 17β-estradiol self-assembled monolayer electrode using a redox marker. *Analyst* 128, 182–186 (2003)

5. Kaneki, N., Xu, Y, Kumari, A., Halsall, H.B., W.R. Heineman & P.T. Kissinger: Electrochemical enzyme-immunoassay using sequential saturation technique in a 20-Mul capillary-digoxin as a model analyte. *Anal Chim Act*a 287, 253-258 (1994)

6. Duan, C & M.E. Meyerhoff: Separation-free sandwich enzyme immunoassays using microporous gold electrodes and self-assembled monolayer/immobilized capture antibodies. *Anal Chem* 66, 1369-77 (1994)

7. Niwa, 0, Y. Xu, H.B. Halsall & W.R. Heineman: Small-volume voltammetric detection of 4-aminophenol with interdigitated array electrodes and its application to electrochemical enzyme immunoassay. *Anal Chem* 65, 1559-1563 (1993)

8. Hayes, F.J, H.B. Halsall & W.R. Heineman: Simultaneous immunoassay using electrochemical detection of metal ion labels. *Anal Chem* 66, 1860-1865 (1994)

9. Nikolelis, D.P, M.G. Tzanelis & U. Krull: Electrochemical transduction of the acetylcholine - acetylcholinesterase reaction by bilayer lipid membranes *J Anal Chim Acta* 282, 527-34 (1993)

10. Wang, J., M. Jiang, A. Fortes & B. Mukherjee: New label-free DNA recognition based on doping nucleic-acid probes within conducting polymer films. *Anal Chim Acta* 402, 7-12 (1999)

11. Miao, Y.Q. & J.G. Guan: Probing of antibody–antigen reactions at electropolymerized polyaniline immunosensors using impedance spectroscopy. *Anal Lett* 37, 1053–1062 (2004)

12. Kanungo, M, D.N. Srivastava, A. Kumar & A. Q. Contractor: Conductimetric immunosensor based on poly (3, 4-ethylenedioxythiophene). *Chem Commun* 680–681 (2002)

13. Li, C.M, C.Q. Sun, S. Song, V.E. Choong, G. Maracas & X. J. Zhang: Impedance labelless detection-based polypyrrole DNA biosensor. *Frontiers in Bioscience* 10,180-186 (2005)

14. Adeloju, S.B, S.J. Shaw & G.G. Wallace: Polypyrrole-based Potentiometric Biosensor for Urea. Part 2: Analytical Optimisation. *Anal Chim Acta* 281,611-627 (1993)

15. Adeloju, S.B, S.J. Shaw & G.G. Wallace: Electroimmobilisation of sulphite oxidase into a conducting polypyrrole film and its utilisation for flow amperometric detection of sulphite. *Anal Chim Acta* 332,145-153 (1996)

16. Aizawa, M, T. Haruyama, G.F. Khan, E. Kobatake & Y. Ikariyama: Electronically modulated biological functions of molecular interfaced enzymes and living cells. *Biosen Bioelect* 9, 601-610 (1994)

17. Kwon, I.C, Y.H. Bae & S.W. Kim: Electric erodible polymer gel for controlled release of drugs. *Nature* 28, 291-293 (1991)

18. Ramanaviciene1, A & A. Ramanavicius: Application of polypyrrole for the creation of immunosensors. *Critical Rev Anal Chem* 32, 245–252 (92002)

19. Fare, T. L, M.D. Cabelli, S.M. Dallas & D.P. Herzog: Functional characterization of a conducting polymer-based immunoassay system. *Biosen Bioelect* 13, 459-470 (1998)

20. Garnier, G, H. Korri-Youssoufi, P. Srivastava & A. Yassar: Enzyme Recognition by Polypyrrole Functionalized with Bioactive Peptides. *J Am Chem Soc* 116, 8813-8814 (1994)

21. Korri-Youssoufi, H, M. Hmyene, G. Garnier & D. Delabouglise: Cation recognition properties of polypyrrole 3-substituted by azacrown ethers. *J Chem Soc Chem Commun* 20, 1550-1552 (1993)

22. Bauerle, P & S. Scheib: Molecular recognition of alkali-ions by crown-ether-functionalized poly(alkylthiophenes) *Adv Mater* 5, 848-853 (1993)

23. Ramanaviius, A.: Electrochemical study of permeability and charge-transfer in polypyrrole films. *Biologija* 2, 64-66 (2000)

24. John, R, M. Spencer, G.G. Wallace & M.R. Smyth: Development of a polypyrrole-based human serum albumin sensor. *Anal Chim Acta* 249, 381-385 (1991)

25. Sadik, O.A & G. G. Wallace: Pulsed Amperometeric Detection of Proteins-Using Antibody containing Conducting Polymers. *Anal Chim Acta* 279, 209-212 (1993)

26. Sadik, O.A, M.J. John & G.G.Wallace: Pulsed Amperometric Detection of Thaumatin Using Antibody-Containing Poly (Pyrrole) Electrodes. *Analyst* 119, 1997-2000 (1994)

27. Aoki, T, M. Tanino, K. Sanui, N. Ogata & K. Kumakura: Secretory function of adrenal chromaffin cells cultured on polypyrrole films. *Biomaterials* 17, 1971-1974 (1996)

28. Ramanavicius, A., K. Habermüller, J. Razumiene, R. Meskys, L. Marcinkeviciene, I. Bachmatova, E. Csöregi, V. Laurinavicius & W. Schuhmann: An oxygen-independent ethanol sensor based on quinohemoprotein alcohol dehydrogenase covalently bound to a functionalized polypyrrole film. *Progr Colloid Polym Sci* 116, 143-148 (2000)

29. Niwa, O, M. Morita & H. Tabei: Electrochemical behavior of reversible redox species at interdigitated array electrodes with different geometries: consideration of redox cycling and collection efficiency. *Anal Chem* 62, 447-452 (1990)

30. Diaz, A.F, G.P. Gardini, W.D. Gill; P.M.Grant, K.K. Kanazawa, J.F. Kwak & G.B. Street: Polypyrrole: An Electrochemically Synthesized Conducting Organic Polymer. *Synth. Met.*, 1980, 1, 329-336

31. Li, C.M, C.Q. Sun, W. Chen & L. Pan: Electrochemical thin film deposition of polypyrrole on different substrates. *Surface & Coatings Technology*, in press (2005)

32. Cosnier, S & A. Lepellec: Poly (pyrrole-biotin): a new polymer for biomolecule grafting on electrode surfaces. *Electrochim Acta* 44, 1833-1836 (1999)

33. Brandl, V & R. Holze: Influence of the preparation conditions on the properties of electropolymerised polypyrrole: *Ber Bunsenges Phys Chem* 102, 1032-1038 (1998)

34. Cosnier, S: Biomolecule immobilization on electrode surfaces by entrapment or attachment to electrochemically polymerized films. A review. *Biosen Bioelect* 14, 443-456 (1999)

35. Skládal, P: Advances in electrochemical immunosensors. *Electroanalysis* 9, 737-745 (1997)

36. Ghindilis, A.L, P. Atanasov, M. Wilkins & E. Wilkins: Immunosensors: electrochemical sensing and other engineering approaches. *Biosenss Bioelectron* 13,113-131 (1998)

37. Kariakin, A.A, O.A. Bobrova, L.V. Lukachova & E.E. Karaakina: Potentiometric biosensors based on polyaniline semiconductor films. *Sensors and Actuators B* 33, 34-38 (1996)

38. Gebbert, A, M. Alvarez-Icaza, W. Stocklein & R.D. Schmid: Rea-time monitoring of immunochemical interactions with tantalum capacitance flow-through cell. *Anal Chem* 64,997-1003 (1992)

39. Li, C.M, S. Song, G. Maracas & V-N Choong: Reporterless Genosensors Using Electrical Detection Methods. *US 20020051975*, 1999 filed (2002)

40. Lee, T-Y & Y-B Shim: Direct DNA Hybridization Detection Based on the Oligonucleotide-Functionalized Conductive Polymer. *Anal Chem* 73,5629-5632 (2001)

41. Li, C.M, J. R. Sawyer, V.E. Choong, G. Maracas & P.M. Zhang: Protein and peptide sensors using electrical detection methods, *US 6,824,669*, November 30 (2004)

42. Bard, A.J. & L. R. Faulkner: Equivalent circuit of a cell. In Electrochemical Methods. Authors: Bard A.J. & L.R. Faulkner, 2nd ed. John Wiley & Sons, Inc. (2001)

43. Rubina, A.Y, V.I. Dyukova, E.I. Dementieva, A.A. Stomakhin,V.A. Nesmeyanov, E.V. Grishin & A.S. Zasedatelev: Quantitative immunoassay of biotoxins on hydrogel-based protein microchips. *Anal Biochem* 340, 317–329 (2005)

44. Crowther, J. R.: Preface. Overview of ELISA in relation to other disciplines. In The ELISA Guidebook: Humana Press (2001)

Key Words: Polypyrrole electrode, Protein Biosensor, Labelless Detection, Impedance Measurements, Immunosensors

Send correspondence to: Dr. Changming Li, School of Chemical & Biomedical Engineering, Nanyang Technological University, Nanyang Avenue, Singapore 639798, Tel: 65 6790 4485, Fax: 65 6792 0451, E-mail: ecmli@ntu.edu.sg

http://www.bioscience.org/current/vol10.htm