Polypyrrole and its nanocomposite based electrochemical immunosensors

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**POLYPYRROLE AND ITS NANOCOMPOSITE
BASED ELECTROCHEMICAL IMMUNOSENSORS**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2008

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Polypyrrole and Its Nanocomposite Based Electrochemical Immunosensors

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School of Chemical and Biomedical Engineering

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Summary

The goal of this research is studies of polypyrrole (PPy), polypyrrole propylic acid (PPa) and PPy nanocomposite based electrochemical immunosensors for high sensitivity and good specificity.

The nanocomposite was electrochemically one-step synthesized to incorporate gold nanoparticles and PPa into PPy matrix for significantly improving the stability and electroactivity of the film. *In situ* atomic force microscopy (AFM) associated with simultaneously electrochemical measurements was employed to study the nucleation and growth process and a mechanism is proposed accordingly.

An impedimetric immunosensor based on the nanocomposite was designed and fabricated, and a novel data processing method was investigated. Normalized impedance was introduced to present the unit impedance change for reducing the variation of the bulk electronic properties of Ab/PPy films and then improving the signal to noise ratio (S/N).

PPa as a component in the sensing composite film provides high porosity and hydrophilicity for sensitivity enhancement, while introducing high density of carboxyl groups of PPa for covalent immobilization of protein probes, leading to significantly improved detection sensitivity compared with conventional entrapment methods. As a model, anti-mouse IgG was covalently immobilized or entrapped in the PPa film and used in a sandwich-type alkaline phosphatase-catalyzing amperometric immunoassay with *p*-aminophenyl phosphate as the substrate, demonstrating remarkable improvement of the immunoassay sensitivity.

The PPy/PPa/Au nanocomposite film was used to construct a label-less impedimetric immunosensor. High density carboxyl groups from PPa were employed to covalently attach protein probes with good orientation and improve hydrophilicity of the produced film. The incorporation of gold nanoparticles significantly improves the electrochemical activity, stability and conductivity of the co-electropolymerized film. Interleukin 5 (IL5) impedimetric immunosensor was fabricated and the detections were successfully performed in both PBS and 1%

human serum with a few pg/ml of detection limit, which is very comparable with the commercially available ELISA methods.

A regenerable PPy/PPa/Au immunosensor was designed and fabricated, which involved immobilization of protein G onto this film as the probe anchor, followed by attaching anti-leptin IgG onto the immobilized protein G as the probe. The regeneration of the immunosensor was achieved by rinsing the electrodes in 0.1M glycine buffer (pH=2.7) for a short period. The binding or dissociation of the anti-leptin IgG to protein G and the detection of leptin were studied by impedance spectroscopy. The great immunosensor performance and regeneration capability were further exhibited in 1% human serum.

This work not only provides fundamental insights of surface chemistry and nanoscience in sensing schemes, but also exhibits great potential in clinical, pharmaceutical and environmental applications due to the high sensitivity and specificity of these immunosensors.

Chapter 1 Introduction

1.1 Introduction

Immunosensors have been in the mainstream of clinical diagnostics since the 1970s because of their capability for direct, sensitive and specific detection of various diseases¹. In recent years, their research and development continues to be fueled by society's demands for high quality health care, assessment of food quality, monitoring of environmental pollution and biological safety. The growing interest in the design of immunosensors aims at fast, economical, portable and sensitive technologies.

Various detection methods have been employed in the immunosensors, including UV-visible absorbance, fluorescence, piezoelectric quartz crystal microbalance, atomic force microscopy and electrochemical techniques. However, most of them cannot perform *in vivo* analysis and real-time monitoring, which are essential features for broad applications.

Electrochemical detection techniques including potentiometric, amperometric and conductimetric methods are very promising in solving the problems encountered in the detection methods discussed above, particularly for portable and implantable immunosensors. The effective combination of electrochemistry and immunochemistry can build the direct and indirect electrical detection of various biological analytes. Indirect electrochemical detection often uses redox-active compounds or labeled enzymes²⁻⁴ to generate electrochemical signals for amperometric detections. Its drawback is that an additional electrochemical label or reporter is required. Direct electrochemical detection is very attractive because it can eliminate the need for the additional labels used in indirect electrochemical detection and is a simple detection mainly related to direct electron transfer or changes of charge density or conductivity from the antibody-antigen (Ab-Ag) bindings

Conductive polymers such as polyaniline, polythiophene and PPy in

electrochemical sensors have been extensively investigated⁵⁻¹⁷, of which PPy is mostly used¹⁸⁻²² because of its superior biocompatibility, simple synthesis, and easy immobilization of various biological compounds. However, for the polymer based electrochemical direct detection immunosensor, the most serious limits are insufficient sensitivity and sensing layer stability. In recent years, the emergence of PPy based nanocomposites has attracted more and more attention from researchers due to their unique properties inheriting from both bulk and nano-size materials. Among various PPy based nanocomposites, metal-polymer nanocomposites are of both practical and fundamental importance due to their useful catalytic activity and enhanced physical properties²³. Thus, the research on fast, convenient and sensitive immunosensors for this thesis is focused on these promising directions.

1.2 Motivations

The significant improvement of PPy based electrochemical immunosensors relies on the advances of efficient bioconjugation of antibody/antigens in the PPy matrix for high sensitivity, novel electrical detection approaches for high signal to noise ratio, and superior PPy derivatives or modifiers for improved electrocatalytic performance. The main motivation of this thesis project is to study the fundamentals involved in immunoassay such as surface science, electrochemistry, material science and nanoengineering, and then to construct highly sensitive and robust PPy electrochemical immunosensors with the guidance obtained from the scientific investigations. Nanoscience and nanotechnology is the ability to synthesize nanostructured materials with superior physical and chemical properties, and design and control the structure of an object at scales with the characteristic dimensions of less than about 100 nanometers. Scientists and engineers from different backgrounds have been working extensively on nanoscience and nanoengineering to explore their fundamentals and broad applications. Novel nanomaterials for biosensor research advance rapidly because they display unique physical and chemical properties from the size effect such as quantum effect, surface effect, and macro-quantum tunnel

effect. These effects are expected to benefit the immunosensor performance by utilizing novel PPy based multi-functional nanocomposites, which can provide biomolecules immobilization capability and enhanced electrochemical stability. Functionalized PPy nanocomposites are intensively investigated on their electrochemical charge transfer, hydrophilicity in aqueous ambient, and conductivity. The mechanisms of these improvements are kinetically studied by utilizing electrochemistry, material science and nanoengineering. Several key functions including probe immobilization, stability and polymer hydrophilicity are thoroughly investigated with PPy based nanocomposites for our electrochemical immunosensor platform. Highly sensitive electrochemical immunosensors are constructed and their important applications are explored.

1.3 Objectives

1.3.1 Leaching-free immobilization of biomolecules

Though the entrapment can be a simple and convenient step to immobilize biomolecules in PPy films, there are still some drawbacks to this method. First, the electrochemical deposition has to be performed in an aqueous solution to keep the activity of the biomolecules. Second, the potential used in deposition has to be kept mild; otherwise irreversible oxidation will happen to the biomolecules. Third, the conditions during deposition must be kept appropriate to avoid a chemical reaction that could alter the biomolecule activity. Fourth, leakage of biomolecules may happen when using entrapment immobilization. Fifth, orientation of immobilized biomolecules may cause the loss of biological activity due to the spatial hinderance which cannot be controlled by entrapment immobilization.

Covalent binding is a better efficient strategy for biomolecule immobilization. It includes the electropolymerization of functionalized conducting polymers and then the attachment of biomolecules to the polymer surfaces by covalent binding. This method allows its precise localization, maintains the recognition properties of the biomolecule and could even lead to its specific orientation. The main advantage of

covalent binding lies in the possibility to use optimal conditions for each step. In particular, the initial formation of polymer films could be performed under conditions (organic solvents, high potential values for the polymerization process) that allow the electropolymerization of specific monomers insoluble in aqueous solutions or to obtain optimum polymer conductivity. The application of covalent binding needs the polymerized polymer film carrying terminal reactive groups, such as NH_2 or COOH . Thus, there is a need to import appropriate reactive groups to the pristine PPy. Furthermore, the generated copolymer should be expected to improve the sensitivity/specificity of the immunosensor through the modification brought by functional groups which can efficiently immobilize the biomolecules specifically and improve hydrophilic properties.

1.3.2 Fabrication of nanocomposite with enhanced properties

Nanoparticles of different nature and size can be incorporated into the conducting polymers, giving rise to a host of nanocomposites with interesting physical properties and important application potential. A large variety of nanoparticles have been utilized for this purpose by using both chemical and electrochemical routes. The design of nanocomposites is targeted to get the best out of two different chemicals, frequently with complementary strengths, to form novel materials with improved properties.

Chemical activity is of the highest importance in functional nanocomposites. Sensors, selective membranes, and electrochemical devices are some important devices based on functional nanocomposites. In this type of nanocomposites, mechanical properties are secondary and the emphasis is on reactivity, reaction rates, reversibility or specificity. The combination of inorganic nanoparticles and organic conducting polymers are highly desired due to the reciprocal complementary properties and reactivities. Electrochemical synthesis of conducting polymers is a well-established technique that can easily vary the synthesis conditions. At the same time it is an efficient technique for incorporating metal nanoparticles into growing

polymer films, giving birth to a number of hybrid nanocomposites.^{24, 25}

By varying the nature of the component materials and the reaction parameters, some of the physical properties namely colloidal stability, magnetic susceptibility, and electrochromic property have been improved which was not possible by a simple mixture or blending. As a result, there is considerable potential for developing new nanocomposite to improve the performance of immunosensors.

1.3.3 Label-free detection and impedance data process

In many designs of electrochemical immunosensors, secondary antibodies able to recognize analyte complex with immobilized receptor were applied. Since antibodies are usually not electrochemically active within the desired potential range, redox-active compounds and/or enzymes that are able to generate electrochemical signals can be applied as labels to generate electrochemical signals that can be applied as labels for indication. However, the major disadvantage of indirect immunosensors is the necessity to apply additional immunochemicals labeled by electrochemical labels; it makes this method more expensive and time consuming, since additional procedures mainly based on incubation with labeled antibodies are essential for indirect detection of analyte of interest. In this respect, 'label-free' immunosensors are more attractive, since such sensors allow measurement without any additional hazardous reagents even in real-time. When using conducting polymers as the immobilization platform of biomolecules, the formation of immobilized receptors and analyte complex changes in capacitance/resistance are recorded. When applying the impedance detection into array, the data from the different spots must be normalized due to the difference among the electrodes, and coated materials. Furthermore, impedance spectroscopy is an extremely sensitive detection technique that can be affected by any surface change, ionic strength, or electrodes arrangement. These parameters are always hard to control during the manufacturing process. The direct effect caused by the differences will decrease the sensitivity and specificity of the immunosensor. Therefore, the data process is

significant in impedance spectroscopy and a solution should be found for improving the immunosensor sensitivity and decreasing the interference of the noise.

1.3.4 Construction of reliable and sensitive human interleukin 5 immunosensor.

Human IL-5 is a vital cytokine produced by CD4⁺ T cells²⁶. IL-5 is originally identified by its activity as a B cell growth factor and IgA-enhancing factor^{27, 28}. Moreover, IL-5 regulates growth, differentiation, activation, and survival of eosinophils from bone marrow into peripheral circulation (e.g. into the lungs following allergen exposure)²⁹. It is involved in a range of allergic reactions and mediates immune reactions against parasites. Therefore, the IL-5 detection has significant biomedical and clinical applications. Traditionally, IL-5 detection approaches include secretion assay, ELISPOT and ELISA^{30, 31}. Secretion assay and ELISPOT are derived from ELISA and have the most similar procedures to ELISA, which needs enzyme labeled second antibody to generate optical signals. These ELISA based analytical methods are expensive, tedious and time consuming because of the requirement of optical detection devices, enzyme labeled second antibody, and long incubation time in ELISA. The available commercialized human IL-5 ELISA kit (Anogen, Ontario, Canada) takes six different reagents and total of 4.5 hours for one test, handled with care. Therefore, there remains the need to develop an economical, convenient and fast analytical tool for IL-5 detection. The emergence of PPy based label-free immunosensors is a promising solution of the requirement in analytical detections. With our endeavors in the PPy functionalization and data analysis of the electrochemical detection, the detection of IL-5 is expected to result in a large improvement from the conventional ELISA and ELISA based techniques.

1.4 Organization

This dissertation is devoted to the study of electrochemical immunosensors based on PPy, PPa and PPy/PPa/Au nanocomposites for protein detection with high sensitivity and specificity.

Chapter 1 consisted of an introduction to the fundamental knowledge and concepts of immunosensors. The motivation and objectives of this research was exhibited in detail.

In Chapter 2, the literature on PPy based immunosensors was summarized. PPy immunosensors were reviewed thoroughly by three aspects, including polypyrrole fabrication, hydrophilicity of PPy and probe immobilization. Furthermore, label-less detection based PPy immunosensor and PPy nanocomposites were reviewed. Perspective and applications were also discussed and proposed.

In Chapter 3, the experiments used in our study were stated in detail. PPy deposition conditions on the different substrates were studied and optimized. The synthesis of Au nanoparticles was introduced. Data analysis of AFM and impedance were described in detail and the characterization methods of polymers and nanocomposites were introduced, respectively. Chemicals and instruments used in our study were also listed, respectively.

In Chapter 4, *in situ* AFM method was employed to study the nucleation and growth mechanism of the electrochemical depositions of PPy and PPy/Au nanocomposite. AFM images, particle analysis of AFM images and current-time measurements at a constant potential for both PPy and PPy/Au nanocomposites were investigated. EIS and contact angle measurements were taken to study the effects of different Au nanoparticle concentrations on PPy/Au nanocomposite stability and hydrophilicity, which were used to further understand the deposition mechanism. In combination of *in situ* nanoscale AFM information and theoretical analysis of current-time transient measurements, a nucleation and growth mechanism was proposed.

In Chapter 5, PPy/Au nanocomposite film was thoroughly investigated. PPy/Au nanocomposite films that were synthesized in the solutions containing different concentration of Au nanoparticles were characterized by CV. The morphologies and stabilities of PPy, PPy/Au, PPy/Pt and PPy/TiO₂ were also compared by scanning electron microscopy and AC impedance spectroscopy, respectively. XPS and TEM

were carried out to verify the Au nanoparticles in the nanocomposite. The electrochemical behavior model of PPy/Au was proposed according to CV conducted in 0.5 M NaCl and CaCl₂ solutions, respectively.

In Chapter 6, a PPy based label-less immunosensor was constructed to monitor Ab-Ag bindings. Several kinds of analytes were used to test the performance of this PPy immunosensor. Both specific and nonspecific binding of the immunosensor was studied. Normalized dimensionless unit concept for impedance data was introduced into this work for the first time. The calibration curve of this immunosensor for AZT detection shows a 10 pg/mL detection limit.

In Chapter 7, a PPa based amperometric immunosensor was constructed. Probes can be attached to the abundant carboxyl groups on the polymer surface or entrapped in the polymer network, leading to fast and reliable amperometric responses. In our study, mouse IgG was selected as a model analyte and its detection is realized in the presence of PAPP using the sandwich mode with rat anti-mouse IgG and sheep anti-mouse IgG (conjugated to alkaline phosphatase) as the primary and secondary antibodies, respectively. The performance of immunosensing with respect to detection sensitivity and reliability was presented and discussed in detail.

In Chapter 8, a PPy/PPa/Au nanocomposite was synthesized and characterized thoroughly. Electroactivity, conductivity and stability of PPy/PPa/Au and PPy/PPa were compared. The ratio of Py to Pa was adjusted to produce different PPy/PPa/Au nanocomposite films in which electroactivity were compared. Furthermore, the component ratio for immunosensor construction was optimized to obtain maximum immunosensor response. The incubation time of the immunosensor was also optimized by using different antibody-antigen pairs. IL5 and its antibody were selected as our model to test PPy/PPa/Au immunosensor performance in both PBS and 1% human serum.

In Chapter 9, the label-less impedimetric immunosensor based on affinity protein G immobilized the PPy/PPa/Au nanocomposite film which can combine the advantages from both PPa and PPy/Au nanocomposites. The regeneration process of

protein G and anti-leptin IgG binding was investigated by EIS. The optimal regeneration time of IgG on protein G/PPy/PPa/Au nanocomposite film was studied by optical and impedance measurements. The leptin detections were carried out in both 0.01 M PBS and 1% human serum to confirm the reproducibility of the regenerable immunosensor.

Finally, in Chapter 10, a general conclusion and the future perspectives will be summarized and proposed, respectively.

Chapter 2 Literature Review

2.1 Immunosensors

Immunosensors have been greatly studied and developed because of the high demand of clinical diagnostics, environmental control and drug discovery. The research in immunosensors is to explore an efficient and reliable transducer that can convert an immunological binding event into a measurable signal with high sensitivity, high selectivity, low cost and simple operation. A large number of materials and detection techniques have been investigated to improve the sensor's performance. The emergence of nanotechnology has attracted increasing attention of researchers on the revolutionary enhancement effects of nanomaterials on immunosensors.

According to the methods of signal transduction, immunosensors can be classified into three types: 1) optical 2) electrochemical and 3) mass detection methods.

Optical immunosensors can employ many different types of measurement techniques including absorption, fluorescence, phosphorescence, Raman, SERS, refraction, and dispersion spectrometry for detections in which chemiluminescence, light absorbance, fluorescence, phosphorescence, light polarization or rotation are measured. Surface plasmon resonance³²⁻⁴⁰ and fluorescent⁴¹ immunosensors are often used in optical immunosensors. Enzyme-linked immunosorbant assay (ELISA) is one of the more typical methods that employ fluorescent signals to detect immunological binding events with high sensitivity and superior specificity. However, the optical immunosensors require expensive instrumentation, tedious operation, experienced operator, and sluggish assay.

Electrochemical detection is a very promising method used in immunosensors. This technique is very competitive to optical detection methods such as fluorescence. Since many analytes of interest are not strongly fluorescent and it is often labor-intensive to tag a molecule with a fluorescent label, electrochemical

transduction can offer a convenient and advantageous alternative. By combining the sensitivity of electrochemical measurements with the selectivity provided by bio-reception, detection limits comparable to fluorescence biosensors are often achievable. In addition, electrochemical methods can provide portable or implantable immunosensors and can conduct *in situ* measurements. Electrochemical detection of immuno-interaction can be performed both with and without labeling. Direct label-less detection can be performed by cyclic voltammetry, chronoamperometry, impedimetry, and by measuring the current during potential pulses (pulsed amperometric detection). These immunosensors have been developed using various substrates, such as silicon, silanized metal, conducting polymer and self-assembled monolayers (SAMs). Electrochemical techniques can provide the widest range of detection capabilities with the least precondition on biological sample preparation and without sacrifices from detection sensitivity and selectivity. The main challenges of electrochemical immunosensors are from reproducibility and reliability.

Another transduction scheme for immunosensors is to measure changes in mass after antibody-antigen bindings. For mass-sensitive immunosensors, quartz crystal microbalance⁴², acoustic waves⁴³ and microcantilever beam⁴⁴ have been used. Quartz crystal microbalancing is a sophisticated method that relies on the use of piezoelectric crystals that can be vibrated at a specific frequency with the application of an electrical signal of a specific frequency. The frequency of oscillation is therefore dependent on the electrical frequency applied as well as the mass of the crystal. Therefore, when the mass increases due to binding of chemicals, the oscillation frequency of the crystal changes and the resulting change can be measured electrically to determine the additional mass of the crystal. Microcantilever beam based immunosensor is another kind of advanced mass detection sensor that was developed with atomic force microscopy in recent years. The surface stress of the receptor coated cantilever side bends when binding of the analyte to the receptors occurs. Thus, the binding signal can be measured by monitoring the stress on the microcantilever.

2.2 PPy immunosensors

PPy has been extensively studied as an efficient matrix and electrode material for immunosensors due to its great potential to enhance response time, sensitivity and versatility. PPy can be one-step synthesized with electrochemical polymerization and has good compatibility with biological molecules in neutral aqueous solutions. It can be efficiently immobilized by various biomolecules with easy control of the spatial distribution of the immobilized species.⁴⁵ The PPy can also provide a 3D electrically conducting structure to connect the electron transfer from biomolecules such as the enzyme, to the electrode.⁴⁶ The electronic conductivity of the conducting polymers changes over several orders of magnitude in response to changes in pH and redox potential of their environment.

PPy has been used to make various types of immunosensors, one of which is amperometric immunosensors. Amperometric immunosensors measure the current signals produced from electrochemically active labels attached to the target or reporter molecules at a specified potential. Anti-human serum albumin IgG incorporated PPy with pulsed amperometric detection in a flow-injection analysis (FIA) system is one of the earliest amperometric PPy based immunosensors, in which the peak current is proportional to the injected human serum albumin concentration in FIA.¹⁹

Figure 2.1 Typical FIA responses obtained using conditions: pulse width=120 ms. HSA concentrations: (a) 50, (b) 25, (c) 15 and (d) 5 mg/L.¹⁹

The similar work by using FIA was also reported by Gooding et al⁴⁷. They

developed an amperometric immunosensor based on antibody entrapped PPy membranes in a flow injection apparatus. The modified electrode is pulsed between -0.2 and +0.4 V versus Ag/AgCl. When an antigen is injected into the flow stream a change in current is observed because the current is due to a change in the flux of ions into and out of the PPy matrix during a pulse. The immunological reaction was reversible because the 200 ms pulse was too short to allow secondary bonding forces to be established.

Figure 2.2 Calibration plot of FIA peak against the logarithm of the antigen concentration. Solid points: the mean and 95% confidence intervals of electrodes prepared with 200 $\mu\text{g/ml}$ (nine electrodes). Open circles: the mean of the responses from three electrodes prepared with 50 $\mu\text{g/ml}$ antibody. Line is a fit to an equilibrium binding model.⁴⁷

Cyclic voltammetry can also be used to monitor the interaction between anti-human serum albumin (anti-HSA) incorporated PPy modified electrode and HSA.⁴⁸ It was found that the anodic peak current of the modified electrode was proportional to the concentration of HSA in the PBS buffered solution.

Figure 2.3 (A) Voltammogram obtained at different antigen concentrations; (—) No antigen; (a) 0.025 $\mu\text{g/L}$; (b) 0.05 $\mu\text{g/L}$; (c) 0.12 $\mu\text{g/L}$; (d) 0.24 $\mu\text{g/L}$; (e) 0.48 $\mu\text{g/L}$. (B) Plot of anodic current versus concentration.⁴⁸

Functionalized PPy film can be a sensing platform for electrochemical detection of antibody-antigen interaction. A photoelectrochemical immunosensor for the determination of anti-cholera toxin antibody based on a photosensitive biotinylated PPy film was generated by electro-oxidation of a biotinylated tris(bipyridyl) ruthenium(II) complex bearing pyrrole groups.⁴⁹ After photoexcitation of this modified electrode potentiostated at 0.5 V vs. SCE, a layer of biotinylated cholera toxin was bound to the functionalized PPy film via avidin bridges. The resulting modified electrodes were used to detect anti-cholera toxin antibody.

Figure 2.4 Calibration curves for photoelectrochemical immunosensors with antibodies ranging from 0 to 200 $\mu\text{g/mL}$: (A) anti CT antibody (■), (B) non-complementary IgG antibody (▲).⁴⁹

Besides conventional IgG, other biomolecules can also be used in the construction of PPy based immunosensors. Ionescu et al⁵⁰ constructed an amperometric immunosensor based on T7 phage which bears a peptide sequence from the virus. The electropolymerization of a phage-amphiphilic pyrrole ammonium mixture previously absorbed on the electrode surface provided an efficient entrapment of phages in PPy film. After incubation with a secondary peroxidase-labeled antibody, the immunosensor were applied to the quantitative amperometric determination of West Nile virus antibody at 0 V via the reduction of the enzymically generated quinone in the presence of hydroquinone and H_2O_2 .

Figure 2.5 Amperometric current response of the immunosensor to the hydroquinone/ H_2O_2 system as a function of WNV-antibody dilution in the range 1:10-1:107. Applied potential 0 V vs. SCE.⁵⁰

Potentiometric method is often used for PPy based electrochemical immunosensors. To construct potentiometric immunosensors, specific antibodies are physically or chemically immobilized on the transducer layer. By applying a constant current on the probe immobilized working electrode, the potential on the working electrode will be changed during the antibody-antigen binding events. This changed potential is directly proportional to the concentration of the analytes reacted with the probe on the working electrode over a certain concentration range. However, potentiometric transducer are seldom used in PPy based immunosensors due to bad sensitivity and stability. Only one PPy based potentiometric immunosensor was found by our literature search. This potentiometric immunosensor that detects enzyme labeled immuno-complexes was formed on PPy coated screen printing gold electrode.⁴ A shift in potential caused by local changes in redox state, pH and/or ionic strength was measured. This immunosensor was applied to detect Hepatitis B, Troponin I, Digoxin and tumour necrosis factor, respectively.

Figure 2.6 Typical calibration curves for three analytes, UTSTM system. (a) HBsAg (streptavidin-coated transducers). (b) Cardiac Troponin I (antibody-coated transducers). (c) Digoxin (antibody-coated transducers).⁴

(iii) Conductometric immunosensors

Conductometric immunosensors employing capacitive or resistant transducers have been used for the real-time and label-free measurement of the antigen-antibody reaction. Conductivity measurements have been employed to develop immunosensors based on ion concentration change by the action of the enzymatic label or conducting polymer. Both pristine PPy and PPy derivatives can be used as the sensing platform in conductometric immunosensors. Ouerghi et al.⁵¹ reported an

impedimetric immunosensor based on N-substituted PPy carrying terminal cyano groups on gold electrodes. The anti-rabbit IgG was immobilized through electrostatic interactions between the terminal cyano groups of the polymer and the hydroxyl groups of the antibody. They found that at bias potential of -1.425 V, the Warburg impedance is negligible and the variation of the impedance caused by specific interaction between immune species can be exploited for sensing purposes.

Figure 2.7 Response curve for antigen obtained for a poly(CEPy) electrode modified by antibodies. Applied potential -1.425 V versus SCE, 0.01 M PBS (pH 7.2) with frequency 0.03 Hz.⁵¹

Anti-luteinising hormone incorporated PPy modified gold electrode was fabricated to monitor the charge transfer when luteinising hormone (LH) was added in by electrochemical impedance spectroscopy.⁵² They found that a decrease in peak polaronic phase angle occurred when a redox cycle was performed on the film following exposure to LH.

Figure 2.8 (A) Calibration for PPy film loaded with poly and monoclonal antibody to luteinising hormone exposed to PB, LH and bovine serum albumin (3 mg/mL); (B) calibration for PPy films loaded with monoclonal antibody to LH.⁵²

Besides conventional electrodes such as gold and glassy carbon electrodes, screen printed carbon electrodes can also be used as substrate for construction of PPy based immunosensors. Anti-BSA incorporated PPy film on the surface of screen-printed carbon electrodes was reported by Grant et al.⁵³. In this work, the capacitive and Faradaic components in the form of Nyquist complex plane impedance plots were differentiated. It was found that the Faradaic real component of the impedimetric response acts as the dominant component of the ac impedance response of anti-BSA loaded PPy films upon exposure to increasing concentrations of BSA.

Figure 2.9 Capacitive and Faradaic impedance changes of anti-BSA loaded PPy to BSA at 1 kHz (capacitive (imaginary) response ($\blacktriangle$), Faradaic (real) response ($\blacksquare$)).⁵³

SPR is a powerful tool to monitor the changes of the refractive index at a solid-liquid interface and can be applied to characterize interactions between biomolecules and polymer films⁵⁴⁻⁵⁶ and the biorecognition process^{57, 58}. Li et al⁵⁹ reported a SPR immunosensor based on permeable PPa film. The results show that the introduction of capture antibody conjugated enzyme not only enhances the current responses but also increases the SPR angle shift. The calibration curves of electrochemical and SPR measurements exhibit a similar dependence on the bulk concentration of antigen. An approximate linear relationship can be obtained by plotting the data in semi-logarithmic reference frame.

Figure 2.10 SPR responses of secondary antibody at various concentrations of mouse IgG (A) binding curves (from the bottom up) at the concentration of 1.0 µg/mL, 1.25 µg/mL, 1.67 µg/mL, 2.5 µg/mL and 5.0 µg/mL mouse IgG; (B) calibration curve of mouse IgG in linear reference frame; (C) calibration curve of mouse IgG in semi-logarithmic reference frame. Measurement at each concentration was repeated three times and the results were averaged.⁵⁹

However, there are some drawbacks in the construction and application of PPy based immunosensing. The degradation of PPy in an aqueous solution could result in a change to the electrochemical properties during detection. This problem becomes more serious when high sensitivity is required. Thus, a robust PPy matrix is expected to be helpful to improve the performance of the immunosensor. For impedimetric immunosensors, enhanced conductivity is favorable because of its label-less nature and simple detection scheme. However, it is a great challenge to increase its signal to noise ratio and to have reliability and reproducibility due to the changes of its impedimetric properties during assay time.

2.2.1 Fabrication of PPy films

PPy can be synthesized by either electrochemical or chemical oxidative

polymerization techniques. Chemical polymerization occurs after the oxidation of pyrrole monomers and oligomers by oxidants to the cation radicals that are recombining and forming the polymeric structure of PPy.⁶⁰

However, chemical synthesis methods are difficult to use for miniaturization, construction of sensor arrays, or the optimization of surface microenvironments because of the difficulty in controlling and forming compact and uniform film on the electrode surfaces. Particularly, the chemical polymerization approach is not convenient for making an electrochemical sensor. Electrochemical polymerization is a better solution for producing PPy films in immunosensor development. It is achieved if an initial electron transfer takes place that permits coupling reactions to occur, leading to additional chain growth as additional electrons are transferred. PPy films are formed by electrochemical anodic oxidation of a monomer in the solution. Either conducting or insulating polymer films that coat the electrode surface are formed according to the need of the experiments. The oxidation of monomer solution under certain conditions results in a film deposited on the surface of the electrode and facilitates the control of growth rate and film thickness by employing electrochemical synthesis. Furthermore, the electrochemical synthesis can allow reproducible film formation with the incorporation of immunological agents in comparison with chemical synthesis.⁶¹ The conventional electrochemical synthesis techniques include potential cycling, fixed potential, pulsed potential, and galvanostatic techniques.

2.2.2 Hydrophilicity of PPy and its derivative polymer

PPy is by far the most extensively investigated conducting polymer for biocoating purposes, especially in the area of biosensor applications. However, due to the hydrophobic nature of the redox-polymer film and the hydrophilicity of electroactive species, the biosensor properties are limited mainly by the substrate diffusion through the polymer film.⁶² For the amperometric detection of immunosensors, the diffusion of the electroactive species is vital to the sensitivity of the sensor, which is strongly related to the hydrophilicity properties of the polymer used for the

immobilization of antibody or antigen. Thus, an improvement of the biosensor performance can be expected by increasing the hydrophilicity of the sensing platform.

The approach that has been used as a means of controlling the properties of conducting polymers involved the synthesis and polymerization of substituted monomers. Pyrrole can easily be modified at the N position through alkylation.⁶³ Such modification can keep its electropolymerization capability, while making it possible to modify drastically the physical and chemical properties of the polymer based on the characteristics of the substituents. N-substituted pyrrole with different functional groups and substituent size began to be studied in the early 1980s.^{64, 65} Since then, various N-substituted pyrroles have been synthesized and studied by varying synthesis conditions. However, those studies focused on the chemical synthesis of the resulting polymers.

In recent years, the studies on biosensors revealed that enhanced hydrophilicity of the sensing layer can improve the performance of the biosensor. Habermüller et al⁶² developed a reagentless biosensor based on osmium complexes covalently bound to a PPy backbone via flexible spacer chains. He found that increased hydrophilicity of the polymer film improved the electron transfer properties and the performance of the biosensor. Pyrrole-lactobionamide monomer was also used as an anchoring polymeric layer and it was found that the pyrrole-lactobionamide monomer markedly increased the permeability of the resulting biotinylated copolymer for the permeation of electroactive species which enhanced the transduction step.⁶⁶ Osmium complex substituted pyrrole derivative was used by Reiter et al⁶⁷ to increase the hydrophilicity and the sensitivity of the biosensor to glucose was largely improved in comparison with the polymer without the improved hydrophilicity.

Figure 2.11 Glucose calibration curves for biosensors with improved polymer film hydrophilicity. (□) Co-polymer of 2 with pyrrole (50 mM pyrrole, 8 mM 2, 10 mg mL⁻¹ glucose oxidase, 50 mM KCl, 0.1 M Tris-maleic acid buffer, pH 7). (●) Co-polymer of 2 with pyrrole-3-carboxylic acid (50 mM pyrrole-3-carboxylic acid, 8 mM 2, 10 mg mL⁻¹ glucose oxidase, 50 mM KCl, 0.1 M Tris-maleic acid buffer, pH 7). Potential pulse profile: 20 pulses: 0 V (applied for 5 s) and 1.5 V (applied for 1 s).⁶⁷

In general, the hydrophilicity can facilitate the hydrophilic proteins immobilization on the sensing layer and the penetration of the hydrophilic target molecules to access the polymer matrix and electrode. Since the carboxylic group is hydrophilic, it is very likely to improve the performance of the biosensor based on pyrrole derivative containing carboxylic group.

2.2.3 Probe immobilization methods

One major concern in developing biosensors is immobilization of the biomolecules. For designing biosensors, electrochemically deposited films have shown a number of advantages over more conventional procedures. One major advantage of using conducting polymers as a sensing platform is the electrochemical polymerization that allows the reproducible and precise formation of a polymer coating over surfaces whatever their size and geometry. It may be possible to

immobilize separately different biomolecules on closely spaced microelectrodes. Furthermore, the electrogeneration of conducting polymers can be easily controlled to produce different thickness and other properties. Thus, conducting polymer is very suitable for bulk manufacturing procedures of the biosensor applications.

A number of techniques to immobilize biological molecules on the electrode surface are available, including adsorption, covalent attachment and entrapment within the polymeric chain.

Adsorption is the simplest way to immobilize biological molecules on the surface of electrodes coated by PPy. Various biological molecules such as DNA⁶⁸, bovine serum albumin⁶⁹ and adrenal chromaffin cells²¹ can be adsorbed to electrochemical deposited PPy.

Entrapment is another frequently used technique to immobilize biological molecules. A favorable approach is the incorporation of antibody or antigen in an electrically conducting polymer layer. This method can form the biosensors during a 'one step' procedure. Usually entrapment of biological molecules within the polymeric chains of conducting polymer is carried out during electrochemical polymerization of monomer in the presence of a conducting polymer.

Covalent binding is superior and has been used for immobilization of biological molecules on the surface of PPy. Covalent attachment of biological molecules resulted in higher biological activity than that obtained by adsorption and is responsible for enhanced stability of the analytical system during continuous measurements. The next very important advantage of this method is ordered molecules orientation, which is especially important for performance of immunosensors.

Table 2.1 Comparison of different immobilization methods for PPy.

Immobilization methods comparison		
Method	Advantages	Disadvantages
Adsorption	Simple, mild conditions, less disruptive to biomolecules	biomolecules linkages are highly dependent on pH, charge, solvent and temperature; insensitive
Entrapment	Simple, polymer growing with the existence of biomolecules	Limited by diffusion, loss of biomolecules activity by leakage or spatial hindrance, mild conditions required
Covalent bonding	Stable, leakage free, good biomolecules orientation, easy to procedure optimization, ideal for mass production and commercialization	Multi-steps needed

In Table 2.1, comparison of different probe immobilization methods for PPy shows that covalent bonding can not only avoid the drawbacks encountered by other methods such as leakage, random orientation but also facilitate the optimization of the procedure. Thus, covalent bonding is highly preferred to be used for constructing immunosensors in our study. It is also necessary to import amino or carboxylic groups in the PPy matrix, since there is no such function group for probe immobilization.

Among the various monomers that can electrochemically polymerize in aqueous, pyrrole derivatives are the most suitable candidates. There are two sites that can import functional groups to pyrrole monomers on nitrogen or β position. Both of them can be applied into constructing film under mild conditions in which biomolecules activity will not be destroyed and have their own advantages and disadvantages, as shown in Table 2.2.

Table 2.2 Comparison of pyrrole derivatives on nitrogen and β position

	Advantage	Disadvantage
Nitrogen substituents	Easy to synthesize; Good solubility in aqua	Steric hindrance; low conductivity
Beta-position substituents	Good conductivity;	Steric hindrance; Difficult to synthesize; bad solubility in aqua

2.3 Label-free detection based PPy immunosensor

In 1991, one of the earliest label-free sensing based on PPy membrane in which anti-human serum albumin is directly incorporated into PPy matrix on a polished Pt electrode was reported by John et al¹⁸. In this work, cyclic voltammetry shows that HSA interacts with PPy/anti-HSA film while no interaction occurs without incorporated anti-HSA. This approach was further developed by Sadik and Wallace¹⁹ in two years later. They used pulsed amperometric to detect HSA on galvanostatically electropolymerized PPy/anti-HSA films. An increased current was observed with increasing HSA concentrations.

Figure 2.12 After incubation $50 \mu\text{gml}^{-1}$ HSA in 0.1 M NaNO_3 , for (a) 10 min and (b) 30 min. Peaks A and B.¹⁸

The response of antibody incorporated PPy film to antigen comes from the intrinsic electrochemical property of PPy. The mechanism of charge generation and propagation during Ag-Ab binding at PPy coated electrodes can be described as:

where PPy^+ and PPy^0 are an oxidized and reduced form of PPy, respectively. A^- and K^+ are anion and counteraction, respectively. According to the above mechanism, the current obtained at the biomolecules immobilized PPy electrodes is from the steps: diffusion of ions to the electrode, charge transfer at the porous PPy interface, migration through the PPy matrix, adsorption-desorption of the biomolecules on PPy interface.¹³ The slow rate of adsorption-desorption process in the last step is considered as the rate determining step which can be controlled by applying electrical potential.⁷⁰

Amperometric method is most frequently used in PPy immunosensors, in which conventionally electrochemically active substances or enzymatic substances are generally used as labels to indicate the antibody-antigen binding event. Alkaline phosphatase, horseradish peroxidase and urease are always used in amperometric PPy immunosensors due to their capability to generate electroactive species. Since the sensitivity of the amperometric sensor for redox compound is normally in the lower micron molar range, this kind of immunosensor is not suitable for the analytes when the concentration is lower than that range.⁵³ For higher sensitivity, amplified amperometric immunosensor is proposed to generate larger signal during detection, in which the concentration of redox-active compound can increase many times to enhance the signal to be detected. However, the major disadvantage of an indirect PPy immunosensor is the necessity to apply an electrochemically active label in the detection, and makes the detection more expensive and time consuming. Thus, a label-free electrochemical detection method is much preferred. It is very useful not

only because it can eliminate the need of labeling for saving cost, but also some patient samples are not able to be labeled, or some antibodies do not have additional epitope, the binding site for the label when sandwich immunoassay is used. The majority of label-free electrochemical immunosensors are based on the changes in charge or conductivities without the need for auxiliary electrochemically active labels during the detection. The formation of immobilized probe molecules or analyte complex changes in capacitance or resistance is recorded.⁴⁷ Potentiometric,⁷¹ amperometric² and conductometric¹⁴ transducers have been used for PPy based electrochemical immunosensors that can conduct label-free detection. For potentiometric transducers, the analytes immobilization induces change in surface potential partly due to electron depletion of the PPy layer. Changes in pH or ionic strength of the solution immediately adjacent to the surface could also explain the change in potential. However, this method is not popular in the literature because of its poor sensitivity, accuracy, precision and stability.⁴ In the conductometric PPy immunosensor, the impedance of the PPy coated electrode can be changed with the analytes immobilization on the polymer surface. The captured analytes can be treated as dopant which result in the conductivity change of the PPy film; thus, an impedance change can be recorded during the detection. The label-free detection is a promising direction for PPy immunosensor development due to its prominent advantages over conventional detection with label needed.

Impedance spectroscopy is an effective method for probing the features of surface-modified electrodes.⁷² A small amplitude perturbing sinusoidal voltage signal is applied to the electrochemical cell, and the resulting current response is measured. Both Faradaic and non-Faradaic impedance in the presence of a redox probe are used as an important electronic transduction means of antigen-antibody binding interactions on electronic elements. Sargent and Sadik⁷³ used anti-human serum albumin incorporated PPy film to detect human serum albumin in pH 7.2 PBS. Effect of low, medium and high phosphate ion concentration on PPy-AHSA film was studied by impedance spectroscopy at a bias potential of 0 V at a frequency range of

100 kHz to 100 μ Hz at 5 mV amplitude. The bias voltage was set at 0 V because at this voltage, the voltammetric data exhibits an anodic peak. During the antibody-antigen binding, Bode plot impedance at different bias potential was studied and compared. R_{ct} was extracted from each film and it was found that 0.4 V is a suitable potential for detection. In this study, values of depression angles at different potentials showed a decrease in angle with the increase of antibody-antigen interaction. They further studied the behavior of PPy-AHSA films at normal and overoxidized states in PBS. Impedance measurements using the antibody-modified electrode showed that larger values of C_{dl} were observed at higher positive potentials with a transition from kinetic control to a diffusion control. At approximately -0.2 V, a very abrupt and dramatic jump in C_{dl} accompanied this transition. This increase in C_{dl} with increasing potential was attributed to changes in the structure of the interface associated with the introduction of the first cationic sites into the film. A decrease in C_{dl} beyond 0 V was observed for the antibody-immobilized PPy electrodes upon exposure to HAS solution due to antibody-antigen binding. It was also found that affinity molecule exposure of PPy films loaded with avidin or antibody does not lead to any significant alteration in the Bode plot. However, following a redox cycle (-0.9 to -0.1 V), following exposure to the appropriate analyte there was a decrease in the peak polaronic phase angle. This study provided a platform for the transduction of binding through measuring peak polaronic phase angle decrease following exposure to the desired analyte and then a redox cycle of the film. This indicates the existence of an alteration in film structure around the bio-affinity molecule following bio-affinity binding.

N-substituted pyrrole was also applied to make label free immunosensing.⁵¹ It was found that at a potential of -1.425 V, the Warburg impedance is negligible and the variation of the impedance caused by the specific interaction between immune species can be exploited for sensing purposes, especially at low frequency. The frequency at 0.03 Hz was used to make the calibration curve and this sensor exhibits a good selectivity as well as a large linear dynamic range, from 600 ngmL⁻¹ to 900

ngmL⁻¹.

From the results obtained by the different authors, it can be concluded that redox polymers have capability as immobilization and sensing layers. When the antibody-antigen binding proceeds atop of the redox polymer, the highly charged protein molecules appear at the interface. This results in the exchange of the counter ions between the redox polymer support and the protein layer that yields a significant change in the electrochemical properties of the polymer.⁷⁴ However, the disadvantage is the relatively low reproducibility, which means that detection results are very dependant on the conditions from various aspects such as ion concentration, polymer storage time, synthesis condition, and electrode differences. Furthermore, electrochemical impedance spectroscopy is an extremely sensitive technique on any interfacial change on the electrode. Thus, the method that can reduce the variation should be found.

2.4 PPy nanocomposites

Over the last few years, a special category of composites termed ‘nanocomposite’ has been studied with growing interest. Nanocomposites composed of nanocrystals/Quantum Dots (QD) and conventional nonconductive polymers were fabricated^{75, 76}. To utilize the electroactive properties and thus accomplish electrochemical bioapplications, attentions turned to a relative new generation of polymers-conducting polymers. Among the conducting polymers, PPy is often chosen for biological applications as an ideal electroactive polymer and has been successfully applied to protein and DNA biosensors^{8, 77}. With intensive studies in conducting polymers, some applications including sensors or electronic devices may eventually require nano-sized components. Incorporation of nanoparticles in conducting polymer matrices may be the simplest way to fabricate nanocomposites. Metal nanoparticles, especially noble metal (Au and Pt) nanoparticles, have been explored. The size-dependant properties of “small” metal particles are known to

yield unique electrochemical, electronic, optical, and catalytic properties⁷⁸⁻⁸¹. Haruta and his co-workers have demonstrated that Au nanoparticles (<10 nm) supported on oxides display high catalytic activity for the chemical and electrochemical oxidation of carbon monoxide (CO) and methanol⁸². However, the instability of metal nanoparticle suspensions in solutions inhibits their applications because of their irreversible aggregation over time. On the other hand, the use of metal nanoparticle building blocks that confines them in conducting polymers for specific nanocomposites is promising⁸³⁻⁸⁷. It has been found that the conductivity and the sensing behavior of PPy can be further improved by imbedding metal particles in the polymer matrix to form a metal-polymer nanocomposite⁸⁸. The PPy/Au nanocomposite was deposited onto glassy carbon (GC) electrode via electrosynthesis⁸⁹. The results show that Au nanoparticles are uniformly distributed in the PPy nanocomposite film and the film exhibits a highly microporous structure with polymer fibrils spanning diameters in a micrometer range. The formation mechanism is proposed that Au nanoparticles might be the nuclei for the pyrrole polymerization when they diffuse onto the electrode surface during electrosynthesis. The nucleation and growth of PPy/Au film are conducted on Au nanoparticle nuclei at the early deposition stage. As a result PPy encapsulates the Au nanoparticles and microporous morphology is produced. The incorporation of Au nanoparticles in the PPy matrix significantly improves electronic conductivity, porous structure and electrochemical stability, especially in a neutral solution. The enhancement renders PPy/Au nanocomposite an excellent candidate for energy storage systems, chemical and biological sensors and electronic devices, and could provide a universal and economic approach for an advanced functional material.

For synthesizing PPy based nanocomposite, chemical synthesis and electrochemical synthesis are available. Chemically synthesized nanocomposites are always in powder form and the parameters of the resulting product are hard to control. In comparison, electrochemical synthesis of conducting polymers is a well established and the conditions such as quantity, morphology and thickness during the

synthesis are easy to control. Furthermore, the films produced by electrochemical techniques can be covered on conducting surfaces with complicated topology, which is significant for industrial manufacturing. Yoneyama et al⁹⁰ successfully incorporated WO₃, SiO₂, SnO₂ and Ta₂O₅ particles into an electrochemically growing PPy film. Kawai et al⁹¹ reported that photoelectrochemical charged TiO₂ particles were inserted into PPy matrix as dopants to form PPy/TiO₂ nanocomposite. Metal nanoparticles also have been used to synthesize PPy based nanocomposites. Dispersed metal nanoparticles in conducting polymers present a promising material for electrocatalytic applications. A good number of works reported conducting PPy/Pt,²³ PPy/Ti,⁸⁷ PPy/Pd and PPy/Ag⁸⁵ nanocomposites. The great potential of these nanoparticles for sensing devices is highly expected. Since enhanced properties are always achieved in these nanocomposites, sensing devices based on these nanocomposites are expected to benefit from these materials.

2.5 Perspective and applications

The use of PPy has been proved to be an effective and promising route for expanding the construction of immunosensors. In the previous studies, it was shown that PPy has the capability to transduce the immunological binding event to an electronic signal either directly or indirectly. Considering the advantages of direct sensing transduction in rigorous clinical and environmental applications, direct ways based on the feature of PPy itself are expected to be favored in future research. The interaction between charged immunological agents and PPy chains results in detectable change in the direct electrochemical signal. A wide range of counterions including various biomolecules, can be used to lay great influence on the ion-exchange behavior of PPy. On the other hand, the functional groups are imported into the traditional PPy matrix to gain more features such as molecules covalent binding or enhanced hydrophilicity. The properties of these groups including chain length and steric structure will influence both the functions and behavior of the

resulted immunosensor.

The advance of nanotechnology makes the delicate control of the molecular structure at the surface of the electrode possible. The certain nano-size structure is promising to help realize the single molecule detection due to the size effect. Various nanostructures including particles, wires, rods, pores, and tubes are being applied to research to obtain higher immunosensor performance. The tailored nanostructured nanocomposites make their biosensors more sensitive and more specific. The biocompatibility and biofunctionality of nanocomposites need to be further investigated for safety, reliability and feasibility in applications. Thus, future endeavors may need to be devoted to the immunosensor applications of PPy based nanocomposites. Up to date, intensive studies are focused on fabrications of different PPy based nanocomposites, but little research is being done in their immunosensor applications. Exploration in this area could lead to important developments in immunosensor research, which are expected to provide better sensing capabilities in future.

Chapter 3 Experimental

3.1 PPy electrochemical deposition

3.1.1 Effect of deposition methods on PPy films

PPy can be synthesized by chemical and electrochemical methods. The advantages of electrochemical deposition of PPy are that films can be prepared simply with a one-step procedure and exact control of thickness. The conventional electrochemical synthesis approaches of PPy film include cyclic voltammetry, constant current and constant voltage depositions. However, different synthesis methods result in different films that are suitable for different applications.

Cyclic voltammetry, galvanostatic and potentiostatic depositions were carried out to investigate the effects of deposition methods on properties of PPy thin films. The thickness of PPy is determined by the electric charge passed during the electrochemical polymerization. The film thickness was made identical to each other for comparison by controlling the same electric deposition charge of 0.42 C/cm^2 . All investigation works on deposition methods were done with glassy carbon electrodes. The CV was conducted over potential range of -0.2 to 1.02 V vs. Ag/AgCl at scan rate of 100 mV/s for five cycles. PPy depositions with galvanostatic and potentiostatic methods were conducted at 0.25 mA/cm^2 for 2 min and at 0.8 V vs. Ag/AgCl for 100 s , respectively. After each deposition, CV in 0.1 M PBS solution was performed to characterize the electrochemical reactivity of the PPy films made from three different deposition methods.

The results are shown in Figure 3.1. Electrochemical reaction activity of an electrode material is the most critical parameter for applications in energy storage systems and electrochemical sensors. In a CV, the higher the redox peaks, the greater the electrochemical reaction activity. Apparently, the galvanostatically deposited polymer thin film had the highest redox peaks, indicating the greatest electrochemical activity, and the potentiostatically deposited film had the worst

redox capacity. Thus, to have highly sensitive electrochemical immunosensors, we use galvanostatically deposition to synthesize PPy in most cases.

Figure 3.1 Cyclic voltammograms of PPy films deposited with different methods: (1) electropolymerization at constant current; (2) CV polymerization; (3) electropolymerization with constant potential; solution: 0.1 M PBS.

3.1.2 Impedance response vs. frequency on PPy films

Impedance spectroscopy is an effective method for characterizing the interfacial properties of electrodes. The complex impedance can be presented as the sum of the real, Z_{re} , and imaginary, Z_{im} components that originate mainly from the resistance and capacitance of the cell, respectively. A plot of Z_{im} vs. Z_{re} over frequencies in terms of impedance measurements, called a Nyquist diagram, is a powerful tool to evaluate an electrochemical system. Figure 3.2 shows impedance results measured in 0.1 M PBS solution at galvanostatically deposited PPy films on DuPont 7102 carbon nanocomposite electrodes with different DC bias vs. Ag/AgCl, but with the same ac amplitude of 10 mV. In Figure 3.2, Nyquist diagrams include a semicircle region lying on the Z_{re} axis followed by a straight line. The semicircle part, observed at higher frequencies, and well-known in literature⁹², corresponds to the electron transfer process, in which the radius of the semicircle is called Faraday resistance, whereas the linear part is characteristic of the lower frequencies range and represents the diffusion-controlled electrode process. However, the smaller the radius of the

semicircle, the lower the Faraday resistance and the faster the electrochemical reactions. All Nyquist diagrams obtained with different electrodes including glass carbon, gold, and carbon nanocomposite electrodes demonstrated the same trend as the typical curve shown in Figure 3.2, indicating that more positive bias had lower Faraday resistance. This showed that the PPy films deposited on carbon nanocomposite electrodes made from carbon inks had the same electrochemical behavior as gold and glass carbon electrodes. For a conjugative conductive polymer, the redox reaction comes from the anions doping/undoping process. The results further indicated that PPy films deposited on different electrodes are p-type conjugative polymer, and can become more conductive and more electrochemically active at more positive polarization in an anodic process.

Figure 3.2 Impedance measurements at galvanostatically deposited PPy film on DuPont 7102 electrode with different DC bias.

3.1.3 Stability of PPy films on different substrate electrodes

In this study, all PPy films were formed by galvanostatic deposition at a constant current of 0.25 mA/cm². The main parameters of the carbon nanocomposite electrodes made from carbon inks are shown in Table 3.1.

Composition and roughness of carbon composite electrodes			
Name	TU-158	Dupont 7102	Dupont 7105
Manufacturer	Asahi	DuPont	DuPont
Binder	phenolic	acrylic	vinyl resin
Binder (%)	35	10–30	10–30
Roughness (nm)	770.6	469.4	869.8

Table 3.1 Composition and roughness of carbon composite electrodes.

The instability of PPy films in aqueous solution are mainly from solution attack, resulting in possible oxidation and swelling for film degradation. The stability experiments were conducted by soaking the deposited PPy films in 0.1 M PBS solution, and impedance measurements were performed in 0.1 M PBS solutions at the start and end of the electrode soaking. Since the sizes of glass carbon and gold electrodes (3-mm diameters) are much larger than that of screen-printed carbon nanocomposite electrodes (100- μ m diameter) and the sizes of screen-printed electrodes had variations from each other, the impedance measured was normalized as change ratio of impedance measured at the end of the soaking to that measured at the start of the soaking for comparison.

The results of resistance measured at 100 Hz at different electrodes are shown in Figure 3.3, which demonstrates that the stability of PPy films on carbon nanocomposite electrodes made from DuPont's inks were better than glass carbon, gold and Asahi carbon ink-made nanocomposite electrodes. Carbon nanocomposite electrodes were rougher and had a much higher specific surface area than glass carbon and gold electrodes. This might make the stability of PPy films on the DuPont carbon surface better than the smooth electrode surface of glassy carbon and gold electrodes since roughness and large surface area could enhance adhesion and enforce the structure of PPy film for stability. TU-158 carbon used different binders from DuPont

7102 and 7105. The binders could possibly affect the PPy deposition. However, the results in Figure 3.3 do indicate that properties of electrode substrate materials affect the stability of PPy films.

Figure 3.3 Stability of galvanostatically deposited PPy films on different substrate electrodes.

The relationship of stability of PPy films on the electrode surface vs. testing frequencies was investigated. The resistance change of PPy films on DuPont 7102 carbon electrode surface was measured after overnight immersing in 0.1 M PBS solutions, and the results were plotted as resistance change vs. frequency as shown in Figure 3.4. The results demonstrate that there was no significant resistance change overnight in 0.1 M PBS from 1 MHz down to 10 Hz, and the resistance change sharply increased below 10 Hz. The results show that the instability occurred at lower frequencies. The resistance at lower frequencies represents the electron diffusion process in the PPy films. In terms of these results, we would suggest that the solution attack did not change the backbone of the conjugative polymer film to affect its doping process, but possibly partially isolated electronic communications between polymer molecules limit the electron diffusional process in the PPy films.

Figure 3.4 Stability of galvanostatically deposited PPy films vs. frequencies in 0.1 M PBS solution.

3.2 Synthesis of Au nanoparticles

In the experiments, Au colloid is fabricated through the chemical reduction. The conventional reductants are phosphorus white, ethanol, hydrogen peroxide, NaB_4 , ascorbic acid, and tannic acid. Au nanoparticles made by tannic acid and sodium citrate is chosen in my work because this method produces homogeneous nanoparticles and the size can be controlled by easily adjusting the reduction concentration. Furthermore, after we compared several different methods, Au nanoparticles made by tannic acid and sodium citrate show the best stability when mixed with pyrrole monomer.

The synthesis of Au colloid was carried out as described by Slot et al.⁹³ In detail of the synthetic work, two solutions were prepared: solution (a) containing 1mL of 1% HAuCl_4 in 79mL deionized water and solution (b) consisting of 155 μL of 1M tri-sodium citrate, 4mL of 1% tannic acid, 0.2mL of 0.1M K_2CO_3 in total volume of 20mL. Both (a) and (b) were brought to 60 °C on a hot plate. Then the solution (b) was quickly added to the solution (a) while stirring. Finally the solution was heated until boiling. The concentration of the Au nanoparticles (about 5.2 nm) is 6.85×10^{-8} M. This colloid can be further concentrated by evaporating the water.

3.3 Synthesis of Pa

In this work, Pa was prepared as follows: 3.8 mL pyrrole (55 mM) and 0.3 mL benzyltrimethylammonium hydroxide were admixed, and then 2.9 mL acrylonitrile (55 mM) was added gradually in order to keep the temperature below 40 °C owing to the strong exothermic reaction. After overnight stirring, the resulting mixture was hydrolyzed by adding 50 mL potassium hydroxide (10 M) and refluxed overnight. The solution was cooled down and HCl was gradually added to acidify the solution to pH 3. The aqueous layer was extracted with ethyl acetate four times, each time with 30 mL solvent. The combined organic layer was washed with 75 mL brine, and then dried with anhydrous magnesium sulfate. After solvent evaporation, the crude product was crystallized by methylene chloride and hexane. About 5 g pure Pa was obtained (65% yield). NMR (CDCl₃): 2.83 ppm (t, 2H, J=7.2 Hz), 4.20 ppm (t, 2H, J=7.2 Hz), 6.14 ppm (t, 2H, J=2,1 Hz), 6.67 ppm (t, 2H, J=7.2 Hz), 9.00 ppm (s, broad peak 1H).

Scheme 3.1 Scheme of Pa synthesis procedure.

3.4 Atomic force microscopy

The surface morphology of the deposited conducting polymers was characterized by a roughness analysis and a bearing analysis. The roughness analysis gives the surface roughness (root-mean-square: R_{RMS}) over a sample by the following equation and R_{RMS} corresponds to the standard deviation of the surface heights (Z_{ij}), where Z_{ave} and N are the average value and the number (256) of Z_{ij} , respectively.⁹⁴

$$R_{RMS} = \sqrt{\frac{\sum_{j=1}^N \sum_{i=1}^N (z_{ave} - z_{ij})^2}{N^2}}$$

The bearing analysis provides a method of plotting and analyzing the histogram of Z_{ij} over a sample. A surface area and a volume above a certain height are calculated.⁹⁴ Since the bearing analysis is very sensitive to the curvature of the image plane, a third-order polynomial plane fit was used to account for the sample tilt and the slight curvature which results from coupling between the z and x-y piezoelectric positioners. The peak in the histogram represents the distribution of z_{ij} and its width (full width at half maximum: W) is a good measure of the surface roughness. If Z_{ij} distributes randomly around the surface height at 0 nm, the peak has a Gaussian shape with the standard deviation R_{RMS} . Thus, W is represented by the following equation and most areas of the peak are in the 2W region between $\pm W$. We have defined the bearing volume (V) as the volume in the bearing curve in the 2W region.

$$W = 2.36 R_{RMS}$$

The bearing volume corresponding to depths more negative than $-W$ is associated with the large particles on the surface. The bearing volume associated with the 2W region from $-W$ to $+W$ will be attributed to the initial surface roughness and small particle formation. Thus, we can get the surface large particles change information with current through the bearing volume negative than $-W$.

The particle analysis is another approach which is related to the bearing analysis and defines particles based on the height of pixel data. This analysis is designed for analyzing well isolated particles. Particles may be analyzed singly or in quantities. Particles in this context are conjoined pixels above or below a given threshold height. The Particle Analysis functions are similar to analyzing grains (Analyze Menu/Grain Size). The analysis includes a histogram of particle size, which can be used to identify specific particles by size. Measurements on this analysis include: the mean area and standard deviation of the particle sizes, the total number of particles, a correlation histogram, a bearing ratio curve and a depth histogram.⁹⁴

3.5 Electrochemical impedance measurement and data analysis

Electrochemical impedance spectroscopy is one of the most sensitive detection methods. Any changes happening on the electrode surfaces such as charge, capacitance or morphology will result in the impedance spectra change.

Figure 3.5 shows a typical shape of a Faradaic impedance spectrum. The semicircle at higher frequencies represents the electron transfer-limited process, whereas the linear part is characteristic of the lower frequencies range and represents the diffusion controlled electrochemical process. Curve b shows the linear curve where the electron transfer process is very fast and curve c shows only the large semicircle spectrum because of the very slow electron transfer resistance. The semicircle diameter equals to the electron transfer resistance, R_{ct} . The intercept of the semicircle with the Z_{re} at high frequencies is equal to the solution resistance R_s . Extrapolation of the circle to lower frequencies yields an intercept corresponding to $R_s + R_{ct}$. The meaningful frequency range for electrochemical systems is between 10 mHz and 100 kHz because of the inductance interference of the electrochemical cell.⁷⁴

However, the high sensitivity of the impedance measurement brings the problem of the noise during the impedance measurements. Any tiny condition change will result in a large alteration of the spectrum. Thus, we need to carefully control the conditions that will affect the impedance measurement and reduce the possible interference. 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 $\Delta R_N = (R_2 - R_1)/R_1$. 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 labelled 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 literature⁷

where $\Delta g = g - g_0$; g_0 is the conductance of the sensor without any interest analyte and g is the conductance of the same sensor in the presence of the analyte. The normalized dimensionless unit resistance change introduced here is based on results from a single electrode. In our experiments, the frequency range is between 100 kHz-0.01 Hz with AC amplitude of 10 mV. Bias potential is varied according to the experiment.

Figure 3.5 Schematic Faradaic impedance spectra presented in the form of a Nyquist plot for: a) A modified electrode where the impedance is controlled by diffusion of the redox probe (low frequencies) and by the interfacial electron transfer (high frequencies). b) A modified electrode where the impedance is mainly controlled by diffusion of the redox probe. c) A modified electrode where the impedance is controlled by the interfacial electron transfer within the entire range of the applied frequencies. The arrow shows the direction of the frequency increase. Resistance of the electrolyte solution, R_s and electron transfer resistance, R_{et} , are shown.⁷⁴

3.6 Chemicals and instrumentation

3.6.1 Chemicals

Anti-leptin IgG, leptin, anti-streptavidin IgG, streptavidin, horse radish peroxidase (HRP) labeled anti-mouse IgG, Anti-axidothymidine (AZT) IgG, axidothymidine, protein G, bovine serum albumin, human serum albumin, 3,3',5,5'-tetramethylbenzidine (TMB), pyrrole, chlorauric acid trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), phosphate buffered saline (PBS, pH=7.4), *p*-nitrophenyl phosphate (PNPP), toluidine blue O (TBO), acetonitrile, pyrrole and glycine were purchased from Sigma-Aldrich. PDMS (SYLGARD 184) was purchased from Dow Corning

Corporation. Rabbit anti-BAP (Bacterial Alkaline Phosphatase) and rabbit anti-gp120 IgG, the outer envelope protein of HIV-I, were purchased from Biodesign and Virostat, respectively. N-(3-Dimethylaminopropyl)- N'-ethylcarbodiimide hydrochloride (EDC), acetonitrile and hydrochloric acid were purchased from Merck. Fetal bovine serum (FBS) was purchased from Fisher. Pa and alkaline phosphatase *p*-Aminophenyl phosphate (PAPP) was homemade. Sodium citrate tribasic dehydrate and potassium carbonate anhydrous were purchased from Fluka. Tannic acid was purchased from Riedel-de Haen. Human IL-5 and anti-IL5 antibody were provided by BD. Alkaline phosphatase-conjugated anti-mouse IgG, chrompure mouse IgG, and biotin-SP conjugated Affinipure sheep anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pa). All solutions were prepared from Milli-Q water with a resistance of 18 M Ω cm.

3.6.2 Instrumentation

- EG&G PaR-273A potentiostat was used to perform parts of the electrochemical experiments.
- Autolab (PGSTAT30, Eco Chemie B.V., Utrecht, The Netherlands) was used to perform parts of the electrochemical experiments.
- 1260 Impedance Gain-Phase Analyzer and 1287 Electrochemical Interface system (Solartron Inc., Houston, Tex.) were used to perform impedance measurements.
- CH Instruments 660B potentiostat was used to perform parts of the electrochemical experiments.
- Field emission scanning electron microscopy (FESEM) (JOEL JSM-6700F) provided the SEM photos.
- AFM (Nanoman, Veeco, Santa Barbara, CA) was used to characterize the morphology of our samples.
- Fourier transform infrared (ATR-FT-IR) spectroscopy was performed by using the Nicolet Magna IR 560 ESP spectrophotometer (Thermo Nicolet, Madison,

WI) incorporated with a “Golden Gate” ATR module (Graseby Specac, Smyrna, GA).

- FTA4000 (First Ten Angstroms, Portsmouth, VA) was used to measure the contact angle.
- TEM (JEOL JEM-2010) was used to show the high magnification photos on our samples.
- X-ray photoelectron spectroscopy (XPS, Kratos AXIS) fitted with an aluminium anode (1486.6 eV) and a 4 keV Ar⁺ ion sputter gun was used to characterize the sample formation.
- Enzyme-Linked ImmunoSorbent Assay (ELISA) was carried out by using Genios plus (Tecan, Boston, MA) at 450 nm.
- Renishaw Micro Raman was used to measure Raman spectroscopy by using the 632.8 nm lines of the He-Ne laser as the excitation source.
- UV-VIS was measured on HITACHI U-2800 double beam system.
- Sputtering was performed by using Explorer 14 (Denton Vacuum, NJ, USA).
- Probe station Summit 11751B-6 system was used to characterize conductivity of the various films in air.

Chapter 4 *In Situ* AFM Study of Electrochemical Synthesis of PPy and PPy/Au Nanocomposite

4.1. Introduction

Conducting polymers have fueled up broad applications such as capacitors, electrochromic devices, field-effect transistors, batteries and sensors^{8, 77, 95-98}, of which PPy has been most intensively studied due to its good stability and excellent electrical properties^{99, 100}. Particularly, PPy can be simply synthesized by one-step electropolymerization¹⁰¹ for various applications⁹⁹.

Different approaches have been studied to electrochemically synthesize PPy¹⁰², in which the monomer units are adsorbed onto the electrode surface resulting in one-electron oxidation to form a pyrrole cation radical followed by coupling with themselves for a chain polymerization. The morphology and chemical/physical properties of the produced film particularly depend on the deposition conditions including the electrolyte nature, the process kinetics, the deposition potential, the dopant properties and the monomer concentration. Various nanoparticles could be incorporated in conductive polymers to improve their conductivity and stability^{85, 86, 103-110}. The kinetics of PPy deposition is one of the most critical factors to affect its physical and chemical properties. Thus, it is important to fully understand the electrochemical deposition process to guide an optimal, controlled synthesis for high quality PPy, while providing fundamental insights to the electrochemical polymerization. *In situ* AFM measurement has been used to study the electrochemical deposition of conducting polymers such as poly(o-methoxyaniline)¹¹¹, polybithiophene¹¹² and PPy¹¹³, but mainly focused on the polymer morphology changes under different polymerization conditions, particularly the effect of the anion doping process¹¹⁴⁻¹¹⁷. However, the electrochemical deposition kinetics has not been directly studied by *in situ* AFM method. Further, the deposition process of Au nanoparticle/PPy nanocomposite, an important

nanostructure material for high performance conductive polymer has not been studied by *in situ* AFM method.

In this work, *in situ* AFM method was employed to study the nucleation and growth mechanism of the electrochemical depositions of PPy and PPy/Au nanocomposites. AFM images, particle analysis of AFM images and current-time measurement at a constant potential for both of PPy and PPy/Au were investigated. AC impedance and contact angle measurements were used to study the effects of different Au nanoparticle concentrations on the PPy/Au nanocomposite stability and hydrophilicity, which were used to further understand the deposition mechanism. In combination of *in situ* nanoscale AFM information and theoretical analysis of current-time transient measurements, a nucleation and growth mechanism is proposed.

4.2. Preparation and characterization

4.2.1 Electrochemical cell construction

For *in situ* AFM study of an electrochemical process, the electrochemical cell design, especially the working electrode is very critical. Our design is shown in Scheme 4.1. The cell is made of PDMS and is designed to allow the AFM tip to be freely moving on the working electrode surface. Au was coated on the surface of silicon wafer slides as the working electrode, of which a highly smooth surface was required for the *in situ* AFM measurements, and achieved by sputtering with 80 sccm Ar ambient at room temperature to coat 5 nm Ti (DC mode, 500 mA for 20 s) as the first adhesion layer and then 200 nm Au (RF mode, 100 W for 600 s) as the top layer. A Pt wire was surrounded the working electrode as the counter electrode for uniform current distribution. Before every experiment, both working and counter electrodes were rinsed in acetone for 5 minutes. An Ag/AgCl reference electrode was used.

Scheme 4.1 Schematic views of the homemade cell used for in situ electrochemical AFM measurements, (a) top view and (b) side view.

4.2.2 In situ AFM measurements

The PPy/Au nanocomposite was electrochemically polymerized at a constant potential of 1.1V in 0.1M pyrrole + 0.15mM Au nanoparticles + 0.1M LiClO₄. For comparison, PPy was also synthesized with the same conditions for PPy/Au but without Au nanoparticles in the monomer solution. The *in situ* AFM measurements were conducted by recording the topography of the surface of the working electrode in a tapping mode during the polymerization, while simultaneously measuring the polymerization current vs. time by a potentiostat (CHI 660B, USA).

4.2.3 Contact angle measurement

Contact angle measurements were conducted on FTA4000 (First Ten Angstroms, Portsmouth, VA). Before measurement, each sample was rinsed in pure water and dried under nitrogen flow, followed by storing in a dry box overnight. For measurement, three different sites were randomly chosen on each film.

4.3. Results and Discussion

4.3.1 FTIR on Au nanoparticles

The synthesized Au nanoparticles used in this work had a mean diameter of 5.2 nm and were protected from aggregation by surface function group –COOH, which was confirmed by FTIR spectrum as shown in Figure 4.1. The FTIR spectrum clearly demonstrates the carbonyl stretching of –COOH group at 1697 cm⁻¹, which is

consistent with the reported characteristic FTIR spectrum of -COOH on the surface of Au nanoparticles^{118, 119}.

Figure 4.1 Selected wavelength FTIR spectra showing the carbonyl stretching frequency of -COOH; (a) solution containing gold nanoparticles and (b) solution without Au nanoparticles.

4.3.2 *In situ* AFM study on the PPy and PPy/Au nucleation and growth process

Figure 4.2 illustrates the AFM images of PPy ((a1), (a2) and (a3)) and PPy/Au ((b1), (b2) and (b3)) measured at different polymerization time (0.5s, 1s and 2s, respectively), SEM images of PPy (a4) and PPy/Au (b4) synthesized with 2.57 C/cm², and depth histograms of PPy (solid line) and PPy/Au (dash line) films ((c1), (c2) and (c3)). The results indicate there is no obvious PPy growth after 0.5 s polymerization since the morphology (Figure 4.2 a1) has no obvious change from the electrode surface before the deposition (data not shown). However, the growing PPy/Au particles after 0.5 s polymerization show clearly on the electrode surface (Figure 4.2 (b1)), indicating a much faster nucleation process of Py/Au than Py at the initial deposition stage. Depth histogram from the AFM images can be used to analyze well isolated particles in single or quantities⁹⁴. Figure 4.2 (c1), (c2) and (c3) show the depth histogram of PPy and PPy/Au film at different growth times. Particle height can be extracted from the histograms at a specific bearing percent according to the data process method described in⁹⁴. Figure 4.2 (c1) shows that at the first 0.5s the particle

heights of PPy and PPy/Au are 14.4 nm and 21.3 nm, respectively, of which the particle height of PPy is the same as that measured on the electrode surface before deposition, but the height of PPy/Au is much larger than that of PPy, further proving that Py/Au has a faster nucleation rate than that of Py at the initial deposition stage. This can be ascribed to the negatively charged Au nanoparticles, which have a much stronger affinity than the neutral Py to be absorbed on the positively charged electrode surface by electrostatic attraction and become nuclei, followed by conjugation with the N position of Py through their COO^- groups¹²⁰ for fast electrochemical polymerization. Alternatively, Py could attach onto Au nanoparticles in the electrolyte and then be absorbed onto the surface through the strong electrostatic attraction for polymerization. After 1 s deposition, both PPy and PPy/Au fully cover the electrode surface. However, the deposited PPy particles with a particle height of 18.1 nm (Figure 4.2 (c2)) are much more uniform than that of PPy/Au with a particle height of 36.5 nm. The result also shows that some of the deposited PPy/Au particles have sizes equal to the PPy particles size but not the dominant composition. This may indicate there are parallel deposition processes on both Py and Au-Py nuclei during the Au-Py polymerization. The morphologies of PPy/Au and PPy after 2 s deposition do not show a distinct difference, and their particle heights increase to 48.6 and 61 nm, respectively. The Root Mean Square Roughness, R_{RMS} of the deposited polymer surface in Table 1 is estimated from the measured AFM images⁹⁴. Clearly, R_{RMS} increases significantly with prolonged polymerization time. The R_{RMS} between PPy/Au and PPy with 0.5 and 1 s deposition time show significant difference; however, they are almost the same after 2 s deposition time. The results are well in agreement with the AFM images discussed above. The SEM images of thick PPy and PPy/Au films deposited for 2.57 C/cm^2 in Figure 4.2 (a4) and (b4) illustrate that the PPy/Au film is much more porous with larger aggregate particles than the PPy film.

Figure 4.2 AFM images of PPy ((a1), (a2) and (a3)) and PPy/Au ((b1), (b2) and (b3)) at different polymerization time (0.5s, 1s and 2s, respectively), SEM images of PPy (a4) and PPy/Au (b4) polymerization of 2.57 C/cm² and depth histograms of PPy (solid line) and PPy/Au (dash line) films ((c1), (c2) and (c3)).

Table 4.1 R_{RMS} values relative to images reported in Figure 4.2

Polymerization time (s)	PPy R_{RMS} (nm)	PPy/Au R_{RMS} (nm)
0.5	2.863	3.745
1	4.846	7.784
2	11.758	10.373

The AFM *in situ* measurements and their analysis clearly show the polymer

deposition process at a nanoscale, which could provide solid evidence for us to propose the mechanisms for different polymer depositions. Based on the discussion above, during PPy/Au synthesis the polymerization starts from Au nanoparticles as the nuclei followed by deposition with both Au nanoparticles and Py as nuclei for prolonged deposition. It is worthy noting that the negatively charged Au nanoparticles can directly be doped into the formed PPy and then become nuclei to initiate new polymerizations. Since there are always two parallel nucleation processes from Py and Au-Py and the Au nanoparticles have stronger affinity to the electrode surface than Py to cause different polymerization rates, the deposition cannot be uniform and could form a very porous structure with large aggregates. However, for PPy polymerization, only Py can be the nucleus. That is why the PPy film is more uniform and dense than PPy/Au film, which is clearly illustrated in the SEM images of PPy and PPy/Au films deposited with 2.57 C/cm^2 in Figure 4.2 (a4) and (b4), showing that the PPy/Au film has larger aggregates of particles and is much more porous than the PPy film.

4.3.3 PPy and PPy/Au nucleation and growth mechanism

It is known that a deposition process could have two kinds of nucleation, instantaneous and progressive, followed by two possible growth models, two-dimensional (2D) and three-dimensional (3D) growth, which can be expressed by a theoretical equation derived by Harrison and Thirsk¹²¹. The polymerization current (i) recorded vs. deposition time (t) during the *in situ* AFM measurements of electrochemical polymerization is used to fit the Harrison and Thirsk equation and is further compared with the theoretical curves of 2D and 3D nucleation and growth as shown in Figure 4.3, in which i_m and t_m are the maximum current and deposition time, respectively. In instantaneous nucleation mechanism, the number of nuclei is constant and they grow at their former positions originated from the bare substrate surface without formation of new nuclei. Hence the radii of the nuclei are larger and the surface morphology is rougher. In progressive nucleation, the nuclei not only grow at their former positions but also form new nuclei to produce smaller nuclei particles,

resulting in a flatter surface morphology. In the 3D growth, the growth rates of nuclei are essentially equal or comparable in the directions parallel or perpendicular to the electrode surface. However, in the 2D growth, the nuclei grow more quickly in the parallel direction than in the perpendicular direction until they meet and overlap¹²². It is clear that after nuclei overlapping (maximum deposition current (i_m)), the fitting curves from the experimental results for both pure PPy and the PPy/Au nanocomposite electrodeposited on Au electrodes could be considered consistent with the theoretical curve of the instantaneous 3D nucleation; however, the PPy/Au deposition deviates partly from the theoretical curve while the PPy deposition is well in agreement with the model. Before nuclei overlapping, the experimental fitting curves for pure PPy and the PPy/Au nanocomposite are quite different, in which the nucleation and growth mechanism for the PPy/Au nanocomposite departs from the instantaneous 3D model. The shorter time to reach i_m and the higher deposition current of PPy/Au nanocomposite deposition shows its nucleation and growth rate is much faster than both PPy and the theoretical one, which is well in agreement with the results obtained from analysis of the *in situ* measured AFM images. Obviously, these differences can be ascribed to the presence of Au nanoparticles in the Py monomer solution. These can further support the mechanism we proposed for the PPy/Au electrochemical deposition. For deposition of PPy/Au nanocomposite, both Au nanoparticle and Py can be the nuclei although the Au nanoparticle is the dominant process due to its stronger absorption on the electrode surface from its negative charge. That is why the PPy/Au deposition deviates from the instantaneous 3D nucleation at the initial deposition stage. After PPy totally covers the Au nanoparticles, the growth of Au/PPy is simply dependent on the Py polymerization. This makes the fitting curve of PPy-Au basically similar to the PPy, which is consistent with the theoretical curve of the instantaneous 3D nucleation. However, the fitting curve of the PPy/Au deposition deviates from the instantaneous 3D nucleation again after prolonged deposition time ($t/t_m > 2.3$) with larger deposition current. This may indicate the deposition of PPy/Au is only controlled in a short period by the Py

polymerization, and then Au nanoparticles become the nuclei again. This can well explain that the morphology of the thick PPy/Au film is different from PPy, because the nucleation of Py/Au plays a critical role in whole film formation.

Figure 4.3 Dimensionless plots of I - t curves for PPy/Au nanocomposite (■) and pure PPy (□) compared with theoretical models for nucleation and growth. Curve a (dashed line) and b (dotted line) represent 3D instantaneous and progressive models, respectively. Curve c (dash-dot line) and d (solid line) represent 2D instantaneous and progressive models, respectively.

4.3.4 Au nanoparticles concentration influence on the PPy/Au nanocomposite film stability

The effect of Au nanoparticles concentration in the precursor solution on the electrochemically deposited film was investigated by impedance spectroscopy, in which the radius of the semicircle over the high frequency range, R_{ct} in Nyquist plot measured in PBS solution can be used to present the Faraday resistance of the electrochemical reaction⁸, which is apparently the doping-undoping reaction resistance, the only electrochemical reaction for the PPy/Au film in PBS. Four different PPy/Au films were electrochemically synthesized in solutions containing 0, 88.2, 264.6 and 529.2 μM Au nanoparticles content and the R_{ct} was measured by immersing the films in PBS for a long period of time. The change of R_{ct} vs. time is shown in Figure 4.4. Obviously, the increase of R_{ct} indicates the reduction of the electroactivity of the polymer film. This could be ascribed to the nucleophilic attack

from ions in the solution¹²³. The R_{ct} increase is irreversible and is an important indication of electrochemical stability of the films. Although R_{ct} of all films displays an increase trend, the electrochemical stability of the PPy/Au nanocomposite film is significantly improved over the PPy film and is continuously enhanced by increase of Au nanoparticles concentration in the precursor solutions, demonstrating that incorporation of Au nanoparticles can effectively retard the degradation of PPy and improve the electrochemical stability in the aqueous solution. It is reported that PPy shows better stability with the insertion of larger anion dopant¹²⁴. Since Au nanoparticles bear negative charge as confirmed in Figure 4.1, they can be considered as very large “anion” dopant in the PPy matrix to stabilize the PPy film. This can further support the observations through the *in situ* AFM measurements that Au nanoparticles can be directly doped into the PPy and further become nuclei for Py polymerization. Thus, the results from *in situ* AFM measurement help us to better understand the electrochemical stability of a conductive polymer and provide guidance for its further improvement.

Figure 4.4 The impedance spectra change of the PPy/Au films prepared in the solution containing different concentrations of (a) 0, (b) 88.2, (c) 264.6 and (d) 529.2 μM Au nanoparticles content. The different color of the curves in each figure presents the different time.

4.3.5 Au nanoparticles concentration influence on the PPy/Au nanocomposite film hydrophilicity

The hydrophilicity of the different PPy/Au films was also investigated by contact angle measurement. In Figure 4.5, the contact angles of the four PPy/Au films are plotted versus the Au nanoparticles content in the precursor solutions, which concentrations were 0, 29.4, 88.2 and 176.4 μM , respectively. With the increase of the Au nanoparticles content, the contact angle of the resulted films decreases, indicating better hydrophilicity. When the concentration of Au nanoparticles content is above 176.4 μM , the contact angles of the nanocomposites cannot be measured because the water drop spreads instantly on the surface. The hydrophilicity improvement could be ascribed to the carboxylic groups of Au nanoparticles in the PPy/Au film. This improvement renders PPy/Au more suitable in the application of biomedical devices where high hydrophilicity for protein adhesive resistance is favored¹²⁵. The superior electroactivity and stability of PPy/Au can provide great potential applications in sensing and energy conversion systems, of which the research is currently being conducted in the author's lab and will be published in separate papers.

Figure 4.5 Contact angles of the different PPy/Au films.

4.4. Conclusions

In summary, *in situ* AFM measurements with simultaneously electrochemical characterization were developed to study the mechanism of both PPy and PPy/Au nanocomposite deposition from a nanoscale observation. Nanoscale information derived from the *in situ* measured AFM images associated with theoretical simulation in terms of the electrochemical characterization is used to explore the deposition mechanism of PPy/Au. It is discovered that Au nanoparticles with negatively charged carboxylic groups can be the nuclei by both adsorption on the electrode surface and doping on PPy for Py polymerization, and thus has faster nucleation and growth rate than Py alone at the early polymerization stage. In addition, the PPy/Au film is formed by both Au nanoparticle and Py nucleation processes. These make the deposition kinetics of PPy/Au deviate from the theoretical instantaneous 3D nucleation model. With the guidance of its deposition mechanism, a superior PPy/Au film with much higher porosity, electroactivity, stability and hydrophilicity could be fabricated, and renders great potential in its applications in electrochemical sensors and energy conversion systems.

Chapter 5 Electroactivity and Stability Comparison of PPy and PPy/Au Nanocomposite

5.1 Introduction

Over the last few years, nanoparticles of noble metals have been studied with growing interest. The size-dependant properties of small metal particles are known to yield particular optical⁷⁸, electrochemical⁷⁹, and electronic⁸⁰ properties. However, the instability of metal nanoparticles suspensions in solutions inhibits their applications because of their irreversible aggregation over time. On the other hand, it is possible to confine nanoparticles into a conducting polymer matrix. Among various conducting polymers, PPy is the most representative one and can be prepared either chemically or electrochemically. PPy also shows remarkable physicochemical properties which contribute to the application in immunosensors⁷⁷, actuators¹²⁶, DNA sensors⁸, ion exchangers¹²⁷ and liquid sensors¹²⁸. Meanwhile, it has been found that the conductivity and the sensing behavior of conductive polymers such as polyaniline and PPy could be further improved by imbedding metal particles into the polymer matrix to form a metal-polymer complex^{88, 129} during the polymerization process. Such metal-polymer nanocomposites are of both practical and fundamental importance. Aside from their useful catalytic activity toward technologically important substrates, these nanocomposites also show enhanced physical and chemical properties²³. Some metal-polymer nanocomposites have already been reported in the literature, such as conducting PPy/Pt²³, PPy/Ag⁸³, PPy/Ti⁸⁷, PPy/Pd⁸⁵ nanocomposites.

This work focuses on the formation of Au nanoparticles-incorporated PPy film and characterization of this novel nanocomposite. The previously reported PPy/Au nanocomposites^{83, 84, 86, 103, 130, 131} are all synthesized by chemical method. The electrosynthesis method of PPy/Au nanocomposite and its characterization in this study has never been reported. This PPy/Au nanocomposite is different from the

previous gold-PPy core-shell nanocomposites which is present as micelles. Due to the different physicochemical nature of the two materials, and their morphology, and structure situation, the novel nanocomposite synthesized in the work demonstrated superior electrical and electrochemical properties in comparison to pure PPy films formed under similar conditions.

5.2 Preparation and characterization

5.2.1 Various nanocomposites synthesis

PPy film was electrodeposited onto the glassy carbon electrode in a 0.4 M pyrrole aqueous solution, using 0.01 M PBS as the supporting electrolyte. PPy/Au, PPy/Pt and PPy/TiO₂ nanocomposites were prepared in the 0.01 M PBS solution containing 0.4 M pyrrole monomer with 0.88 mM Au nanoparticle content, 5 mM Pt nanoparticle content and 1 mM TiO₂ nanoparticle content, respectively. The electrochemical depositions of all the films were carried out by using galvanostatic deposition with a current density of 1.5 mA/cm² for 5 min. All the deposition solutions were deaerated by bubbling with nitrogen before use. For the SEM investigation, the films were peeled off from the electrode and dried in the air. Galvanostatic deposition was chosen since this technique produces the highest electrochemical reactivity compared with cyclic voltammetry or the potentiostatic deposition method¹⁰¹.

5.2.2 Stability characterization

The stability measurements of the nanocomposites were performed in 0.01M PBS by impedance spectroscopy. After electrochemical deposition of the nanocomposites, the electrodes were washed for three times and then measured impedance continuously at 0, 0.5, 1.5, 4, 6, 9, 13, 19 and 27 hours in 0.01M PBS.

5.2.3 Conductive AFM characterization

Conductive AFM was carried out to characterize the conductivity of PPy and

PPy/Au nanocomposites by using contact mode measurement. DC bias of -0.5V to 0.5V is applied to the tip with a scan rate of 0.2491 Hz, and the PPy and PPy/Au films were held at ground potential on the AFM holder.

5.2.4 TEM sample preparation

Electrochemically deposited PPy and PPy/Au film were peeled off from the glassy carbon electrode and then ground in agate mortar to make small particles. Acetone was added into the mortar to evenly disperse PPy or PPy/Au particles followed by immersion into copper mesh. After acetone evaporation, the copper meshed samples were ready for TEM measurement.

5.3 Results and discussion

5.3.1 Chronopotentiometry of PPy/Au

Figure 5.1 shows the chronopotentiometry deposition curves of PPy on glassy carbon electrodes. The electrochemical deposition was carried out in six aqueous pyrrole solutions (from a to f) containing Au nanoparticle content of 0 mM, 0.0735 mM, 0.147 mM, 0.294 mM, 0.588 mM and 0.882 mM, respectively. When the Au nanoparticle content reaches 0.882 mM, the precursor solution became unstable and black precipitation appeared in 5 minutes. There is no difference between sample a and b which contains very low Au nanoparticle content. However, with the increasing of Au nanoparticle content in the precursor solution, the deposition potential of PPy decreased accordingly. It suggests that the existence of Au nanoparticles can facilitate the electrochemical formation of PPy film. When Au nanoparticle content reached 0.882 mM, the deposition potential under this condition became higher than that of containing 0.588 mM at the beginning stage. The reason should be that some of the Au nanoparticles form precipitation in pyrrole solution which lowers the content of Au nanoparticles and pyrrole monomer.

Figure 5.1 Chronopotentiometry deposition curves of the aqueous pyrrole solution with Au nanoparticle content: (a) 0 mM, (b) 0.0735 mM, (c) 0.147 mM, (d) 0.294 mM, (e) 0.588 mM and (f) 0.882 mM.

5.3.2 Cyclic voltammetry of PPy/Au

The electrochemical behavior change of PPy/Au films formed in the solution containing different Au nanoparticle content was investigated by cyclic voltammetry. A potential range of -0.8 to 0.4 V versus Ag/AgCl reference electrode at scan rate of 50 mV/s was applied. The reduction peak of sample between -0.6 and -0.8 V could be assigned to the Na^+ insertion accompanied by the hydrogenation of $\beta\text{-C}$.⁸⁹ Another reduction peak of PPy between 0.3 and -0.5 V is attributed to the Cl^- dedoping from the positively charged conjugated chain. The oxidation peak between 0.2 and -0.2 V is assigned to the Cl^- doping. With the increasing of the Au nanoparticle content in the solution, the reduction peak between -0.6 and -0.8 V shifts positively. It can be seen that the existence of Au nanoparticles facilitates the insertion of Na^+ in the reduction process of PPy. This result can be explained by the very porous structure of PPy/Au that lowers the resistance of ions shuttle through the polymer matrix.

Figure 5.2 CV of the PPy/Au films in 0.01 M PBS that were electrochemically deposited in the solution containing Au nanoparticle content: (a) 0 mM, (b) 0.0735 mM, (c) 0.147 mM, (d) 0.294 mM, (e) 0.588 mM and (f) 0.882 mM.

5.3.3 Raman of PPy/Au nanocomposite films

Figure 5.3 shows the Raman spectra of PPy/Au films which are electrodeposited from the precursor solutions containing different Au nanoparticle content. As reported in the literature¹³²⁻¹³⁴, the peak in the range of 1520-1620 cm^{-1} represents the C=C backbone stretching of PPy that is related to the conductivity of PPy, and the smaller the wavenumber, the better the conductivity. In Figure 5.3, the peak positions of the C=C backbone are 1576.52 (a), 1580.18 (b), 1580.39 (c), 1581.61 (d), 1582.19 (e) and 1583.83 (f) cm^{-1} for these PPy/Au films synthesized from the solution containing the Au nanoparticle content from 0.294 mM to 0 mM. This indicates that the increased Au nanoparticle content will enhance the resulting PPy/Au conductivity by promoting the C=C backbone. Furthermore, the peak at about 930 cm^{-1} is assigned to the symmetric stretching mode of ClO_4^{-1} dopant. The broader Raman peaks of PPy appearing in the range of 1000-1100 and 1300-1400 cm^{-1} are assigned to the C-H in-plane deformation and the ring stretching, respectively.

Figure 5.3 RAMAN spectra of PPy/Au films which are electrodeposited from the precursor solutions containing 0.294 mM (a), 0.2646 mM (b), 0.1764 mM (c), 0.0882 mM (d), 0.0294 mM (e) and 0 mM (f) Au nanoparticle content.

5.3.4 Electrochemical active surface area of PPy/Au

To further investigate the hydrophilicity of PPy/Au nanocomposites, cyclic voltammetry was also carried out in the 5 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ on these nanocomposites as shown in Figure 5.4. Both the anodic and cathodic peak current from redox of the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ increase with the Au nanoparticle content. The active surface of the PPy/Au nanocomposite modified electrodes calculated by Randles-Sevcik equation shows that PPy/Au nanocomposites have 1.467×10^{-2} , 1.468×10^{-2} , 1.637×10^{-2} , 1.794×10^{-2} and $1.984 \times 10^{-2} \text{ cm}^2$ active surface area that increase with the Au nanoparticle content. This result suggests that the incorporated Au nanoparticles can contribute more active surface area to PPy. This enhancement may come from the improved permeability of the PPy/Au nanocomposites to electrochemical active species. This feature is of importance when PPy/Au nanocomposite is applied to the biosensor development because the analytes or reporter molecules can get through the nanocomposite more easily.

Figure 5.4 Anodic peaks (A) and cathodic peaks (B) from cyclic voltammetry in 5 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ of PPy/Au films.

5.3.5 TEM and SEM analysis

The results of TEM and SEM analysis are shown in Figure 5.5. The TEM images (Figure 5.5a and b) were obtained from a PPy/Au nanocomposite film synthesized in 0.88 mM Au colloidal suspension + 0.4 mM pyrrole monomer solution. The Au particles manifest as the small dark spots in the TEM image. The nanoparticles with a mean diameter of ca. 5.2 nm demonstrate no aggregation and fairly even dispersion except two large size particles were observed, which were formed from the overlapping of several small particles in the synthesis of PPy/Au film in Figure 5.5a.

Figure 5.5b shows the moiré patterns of Au nanoparticles. It exhibits a one-dimensional fringe lattice due to moiré interference, indicating that these Au nanoparticles are in crystalline.

The morphology of PPy/Au nanocomposite was investigated by SEM. In Figure 5.5c, the typically granular raspberry morphology of pure PPy is presented¹²⁴. However, the PPy/Au nanocomposite film exhibits a highly microporous structure with polymer fibrils spanning the micrometer diameter range (Figure 5.5d). The surface morphology change of PPy/Au is ascribable to the existence of Au nanoparticles in the electropolymerization process. It demonstrated that during the electrosynthesis, the Au nanoparticles were as the nuclei for the polymerization of the pyrrole and facilitated the polymerization process. Thus the PPy encapsulated the Au nanoparticles and microporous morphology was produced. This highly microporous structure of PPy/Au nanocomposite suggests the very large surface area and more ion exchange or larger molecule accessibility to the inner PPy/Au matrix in solution in comparison with pure PPy film. Our experimental results further demonstrated that with the increase of Au colloid concentration, the PPy/Au nanocomposites became more porous. This phenomenon is consistent with previous observation in electropolymerization that Au nanoparticles indeed participated in the electrosynthesis.

It is worthy a note that the change of surface roughness of the PPy/Au film against the Au content related to the nano-topology of the film, which is related by its formation mechanism discussed in Chapter 4. A higher concentration of Au nanoparticles in the deposition solution causes more porous and aggregated nano-topology, resulting in higher surface roughness.

Figure 5.5 (a) TEM micrographs of PPy/Au nanocomposite films. (b) Detail of Au nanoparticles embedded in PPy after electrochemical polymerization. (c) SEM image of pure PPy film. (d) SEM image of PPy/Au nanocomposite.

5.3.6 Raman spectra comparison of the PPy and PPy/Au films

Figure 5.6 shows the Raman spectra of PPy film and PPy/Au nanocomposite. Obviously, the relative intensity of the peaks of PPy/Au nanocomposite is much higher than pure PPy film. This significant change on peak intensity is due to the effect of SERS which should result from two aspects: the nanoscale roughness^{135, 136} in PPy/Au film structure and the facilitation of charge transfer¹³⁶ between pyrrole nitrogen and embedded gold nanoparticles. As reported in the literature^{132, 133}, the peak in the range of 1520-1620 cm^{-1} represents the C=C backbone stretching of PPy that is related to the conductivity of PPy¹³⁴. The peak position shift of C=C backbone from 1584.2 to 1572.5 cm^{-1} for pure PPy and the PPy/Au nanocomposite, respectively, shows that the conjugation length is shortened in PPy/Au

nanocomposite, thus a better conductivity can be expected for PPy/Au film. The peaks located at 1051.7 and 1055.8 cm^{-1} for PPy and PPy/Au film are assigned to be the C-H in-plane deformation of oxidized PPy¹³⁷, respectively.

Figure 5.6 Raman spectra of (a) pure PPy film and (b) PPy/Au nanocomposite.

5.3.7 Electrochemical behaviors of the PPy/Au nanocomposite

The electrochemical behavior of PPy/Au and its comparison with pure PPy film were investigated by cyclic voltammetry. The potential range of -0.9 to 0.4 V vs. Ag/AgCl at a scan rate of 100mV/s was used in all CV experiments. Both PPy/Au and PPy films were stabilized in 0.5 M sodium chloride solution by carrying on several scans in the same potential range before the further investigation.

Li et al¹²⁰ suggested that there are two different doping sites in pure PPy film, which are on positively charged conjugated chain (site 1) and on the β -C of the py unit (site 2), respectively. In our study, two reduction processes of the PPy film in the potential range from 0.2 to -0.9 V were distinguished in the cyclic voltammogram, represented by two reduction waves (Figure 5.7a). This result is very similar to the previously reported reduction process of PPy film in neutral 0.5

M NaNO₃ solution⁸⁸. Therefore, the reduction peak of PPy in the potential range of -0.5 to -0.8 V can be assigned to the insertion of Na⁺ cation accompanied by the hydrogenation of site 2 for maintaining electrical neutrality during the reduction process^{129, 138-140}. Another reduction peak of PPy in the potential range of 0.3 to -0.5 V results from the dedoping process of Cl⁻ from site 1. Since only one oxidation peak was observed for PPy film, thus oxidation wave of the PPy film should correspond to the two reduction processes for the doping/dedoping redox reactions (Figure 5.7a), and non-symmetric reduction/oxidation waves occurred. In this study, significant differences between PPy and PPy/Au films in the shape and positions of the redox waves were observed (Figure 5.7a). The oxidation peak current of PPy/Au film is about 0.3 mA, which drops largely in comparison with that of pure PPy film. Furthermore, two oxidation peaks appears in the potential range of -0.4 to 0.2 V and no reduction peak appears in the potential range of -0.5 to -0.8 V in the cyclic voltammogram of PPy/Au film. To find out the origin of the redox peaks in PPy/Au film, CVs of PPy/Au and PPy in 0.5 M CaCl₂ solution were performed. In Figure 5.7 b, it was observed that the oxidation peak and reduction peak of PPy/Au in the potential range of -0.4 to -0.2 V disappeared in CaCl₂ solution. The reduction process of PPy in the potential range of -0.5 to -0.8 V also did not occur and the oxidation peak current of PPy obtained in CaCl₂ decreased to 0.45 mA from about 0.6 mA obtained in NaCl solution. The disappeared reduction process of PPy has been reported to be attributed to the existence of Ca²⁺ because bivalent cations can hardly be inserted into the site 2 reduction process of PPy¹²⁰. Therefore, the inhibition of the site 2 reduction process would lead to the drop of the oxidation peak current of the same site. The same mechanism can be used to explain the cyclic voltammogram changes in PPy/Au film. In Figure 5.7 b, the redox process of PPy/Au in the potential range of -0.4 to -0.2 V did not happen in CaCl₂ solution. Thus, these two redox peaks should represent the redox process of the site 2 and another oxidation peak in -0.2 to 0.2 V can be assigned to the redox process of the site 1 in PPy/Au film.

Figure 5.7 also shows that electric charge of the PPy/Au film generated by the doping/dedoping process was less than that of PPy film. This might indicate that the nanoscale Au particles inserted into the doping sites reduced the reaction centers for doping/dedoping redox reactions. In the PPy/Au nanocomposite, Au nanoparticles could occupy the positions on nitrogen atoms in the Py unit, which would retard the counter anion insertion on site 1. This could be used to explain why the decrease of oxidation peak of the site 1 was observed in Figure 5.7 a. For the redox reaction of site 2, the reduction process shifted from the potential range of -0.8 to -0.5 V to the potential range of -0.4 to -0.1 V and the oxidation process separated from the main oxidation peak in the potential range of -0.2 to 0.2 V to a more negative position which is in the potential range of -0.4 to -0.1 V. This result indicates that the redox reaction of site 2 has been facilitated after the insertion of Au nanoparticles. In addition, both redox peaks of PPy/Au are more symmetric than those of PPy, demonstrating that the incorporation of Au nanoparticles enhanced the reversibility of the doping/undoing redox reactions of PPy.

Figure 5.7 (a) Cyclic voltammograms of PPy and PPy/Au films in neutral aqueous 0.5 M NaCl solutions at a scan rate of 100 mV/s. (b) Cyclic voltammogram of PPy and PPy/Au films in neutral aqueous 0.5 M CaCl₂ solution at a scan rate of 100 mV/s.

5.3.8 Conductivity comparison of PPy/Au and PPy by conductive AFM

The conductivity comparison of PPy and PPy/Au was performed by conductive

AFM in the air at room temperature. The voltage sweep was between -0.5 and 0.5 V and the scan rate is 0.2491 Hz. Figure 5.8b shows that C-AFM current of PPy/Au increased sharply during the voltage sweep in comparison with Figure 5.8a. The noise in the plots should come from the charge repulsion between the AFM tip and films as the noise increases with the increase of voltage on both positive and negative directions. This result from conductive-AFM is very consistent with the RAMAN measurement.

Figure 5.8 Conductive AFM investigations on the comparison of (a) PPy and (b) PPy/Au film.

5.3.9 Morphology of PPy, PPy/Au, PPy/Pt and PPy/TiO₂

The morphology of the nanocomposites was investigated by SEM. Figure 5.9 (a) shows a typical granular raspberry morphology of the pristine PPy grown by electrochemical deposition¹²⁴. In Figure 5.9(b), PPy/Au nanocomposite exhibits a highly micro porous structure with polymer fibrils spanning the micrometer diameter range. This unique surface morphology of PPy/Au is ascribable to the existence of Au nanoparticles in the electrosynthesis process. The Au nanoparticles might be the nuclei for the pyrrole polymerization when they diffused onto the electrode surface. Figure 5.9(c) shows that the PPy/Pt nanocomposite has a similar structure in comparison with the pristine PPy film. More porous sphere structure can be observed due to the incorporation of Pt nanoparticles in the PPy matrix. In Figure 5.9(d), PPy/TiO₂ nanocomposite exhibits particularly denser and more compact morphology, which is consistent with the previous report¹¹⁰. Comparing with PPy, PPy/Pt and PPy/TiO₂, PPy/Au has the most porous structure, which means it has an enlarged surface area for chemical reaction or physical interaction. Figure 5.9(e) displays the existence of Au in the PPy/Au nanocomposite with Au 4f peaks in XPS spectra. Meanwhile, the elements concentration in this PPy/Au nanocomposite is given in Table 5.1. The Au nanoparticles diameter distribution was investigated by TEM in Figure 5.9(f). The histogram shows that the Au nanoparticles have a mean diameter of 5.2 nm and even distribution in PPy/Au matrix.

Figure 5.9 (a) SEM image of the pristine PPy film. (b) SEM image of the PPy/Au nanocomposite. (c) SEM image of the PPy/Pt nanocomposite. (d) SEM image of the PPy/TiO₂ nanocomposite. (e) Au 4f XPS spectra of PPy/Au nanocomposite. (f) Histogram of the incorporated Au nanoparticles diameter distribution in PPy/Au nanocomposite.

	O	N	C	Au
Atomic	21.89	8.46	69.54	0.12
Concentration %				
Mass Concentration	26.39	8.93	62.96	1.72
%				

Table 5.1 Elements concentration in the PPy/Au nanocomposite.

5.3.10 Electrochemical stability comparison among four films

The stability of the polymer is an important factor for some applications such as batteries and chemical and biological sensors. Development of robust PPy films stable in an aqueous solution is essential for better performance of the applications. The stabilities of PPy, PPy/Au, PPy/Pt and PPy/TiO₂ nanocomposites in 0.01 M PBS solution were investigated by measuring their impedance change at 0, 0.5, 1.5, 4, 6, 9, 13, 19 and 27 h, and the results are shown in Figure 5.10. It is obvious that PPy/Au, PPy/Pt and PPy/TiO₂ nanocomposites have much lower resistance in comparison with the pristine PPy film. Of the three nanocomposites, PPy/Au has the best conductivity and stability.

Figure 5.10 The electrochemical stability comparison of PPy, PPy/Au, PPy/Pt and PPy/TiO₂ nanocomposites in 0.01 M PBS.

5.4 Conclusions

In this work, PPy/Au nanocomposite films were synthesized using a simple electrochemical polymerization technique. This synthesis approach can be easily extended to the preparation of a wide range of nanostructure metal/conductive polymer nanocomposites. The resulted film is a nanocomposite with an Au core covered by PPy coating. TEM and SEM investigations showed that Au nanoparticles had a vast effect on the PPy growth process. Au nanoparticles could incorporate into the PPy matrix with their own size unchanged, while making the PPy film more porous and conductive. The result of Raman spectroscopy indicates that the C=C backbone in PPy/Au film was shorter than that in PPy film. Incorporated Au nanoparticles showed the inhibiting effect on the doping/dedoping process of the site 1 in the pyrrole unit. However, Au nanoparticles increased the electroactivity. The PPy/Au nanocomposite shows much better stability than the pure PPy does. As far as the possible applications of such improved electroactive

thin films are concerned, their potential use as sensing layers in devices such as gas or chemical sensors is readily apparent and the work is in progress for this direction. Moreover, PPy, PPy/Au, PPy/Pt and PPy/TiO₂ were compared for their morphology and stability. The results show PPy/Au is the most porous and stable film among these films.

Chapter 6 PPy based Impedimetric Immunosensor

6.1 Introduction

Immunoassay has become a powerful analytical technique since the 1970s due to its capability of direct, sensitive and specific detection in diagnostic applications¹. Accordingly, electrochemical immunoassay has steadily grown greatly and made significant advances¹⁴¹⁻¹⁴⁸ because its measurements are simpler, less expensive, and even more precise than optical detection methods. Conductive polymers such as polyaniline, polythiophene and PPy in electrochemical sensors have been extensively investigated^{5-8, 10-15, 17, 149, 150}. PPy is one of the conductive polymers mostly used in electrochemical immunosensors¹⁸⁻²² because of its superior biocompatibility, simple synthesis, and easy immobilization of various biological compounds. PPy thin films can be electrochemically deposited on different electrode substrates by cyclic voltammetric, galvanostatic and potentiostatic methods^{61, 77, 101, 151-153}. 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 product⁷⁰. Direct label-less 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 solutions¹⁵⁴. The potentiometric method can offer a label-less detection, in which the surface potential changes on an ion selective electrode are used to detect the ionic product of an enzymatic immunoassay reaction. Typically, potentiometry is less sensitive and more slowly responsive than amperometry, and is limited by fewer enzymatic reactions that can be employed by the method⁷¹. Capacitive measurements have been used for the real-time and label-less detection of Ab-Ag interactions.

Generally, the method is limited to detecting relatively large molecules, and requires more precision of capacitance measurements with commercial instruments¹⁵⁵. Impedance label-less detection for DNA hybridization has been reported by Li^{156, 157} and Lee¹⁵⁸. Li and coworkers further reported the impedance label-less detection of protein bindings in a US patent¹⁵⁷. 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, label-less or reporterless electrochemical immunosensors become extremely attractive. The PPy immunosensors including the label-less methods reported so far were not sensitive and demonstrated detection limits from ng/ml to $\mu\text{g/ml}$ range. There remains a need to develop less expensive, more sensitive and reproducible PPy based immunosensor, particularly the label-less immunosensors for practical medical diagnostic applications. In this paper, we reported the advantages of a new impedance label-less 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.

6.2 Preparation and characterization

6.2.1 Electrodes and silicon array chip preparation

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 0.05 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, the electrodes were immersed in 0.5 M sulfuric acid for 5 min., and again washed vigorously with deionized water. Silicon array chips, shown in Figure 4.1a, 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 $100 \times 100 \mu\text{m}^2$ gold working electrodes are 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 6.1b).

Figure 6.1 Silicon array chip. 1a: Silicon chip 1b: Schematic of the electrochemical cell structure.

The surface area of the top electrode is larger than the bottom array electrodes by 1 order of magnitude. In ac impedance 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 procedure used for the glassy carbon electrodes, but without polishing.

6.2.2 Measurements procedure

For immobilization of probe proteins into the PPy film matrix, a 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 $150 \mu\text{g/ml}$ 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 held 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 PPy film. The AC impedance baseline of the PPy immunosensor prepared was first measured 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.

Without chemical cross-linking, the driving force for the entrapment immobilization is dependent on the nature of proteins. If the protein is negatively charged, it is a large “anion” and then can be doped on the PPy chain by electrochemical oxidation. Otherwise, the driving forces for the protein adsorption come from weak interaction forces between protein and PPy molecules such as hydrophobic interaction, hydrogen bonds, π - π interaction, and dative bonding. Since all these driving forces including the doping process are very weak, the denaturation of proteins normally cannot occur. However, the pH of the deposition solution should be controlled around 7 to avoid the protein denaturization.

6.3 Results and discussions

6.3.1 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/PPy 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 PPy/AZT glassy carbon electrodes is shown in Figure 6.2. 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 circuit³³ 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_{ct} , and Warburg impedance, Z_w , a resistance from the diffusion of ions from the bulk electrolyte to the electrode surface. The impedance measured at the anti-AZT/PPy electrode after incubation in AZT/PBS (curve 2 in Figure 6.2) shows significant change than that before the Ab-Ag interaction (curve 1 in Figure 6.2). The diameter of the semicircle apparently increased after the Ab-Ag interaction, indicating R_{ct} , the electrochemical reaction resistance increased. It has been reported that the increase of R_{ct} was due to the hindrance of the hybridized DNA to the anionic doping process of PPy, which was used for label-less detection-based DNA sensors⁸. The larger the diameter of the semicircle is, the higher the R_{ct} is. 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/PPy matrix could create the same steric obstruction to the ion doping and thus, R_{ct} is increased after incubation shown in Figure 6.2.

Figure 6.2 Impedance complexes at the anti- AZT/PPy 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.

It indicated that the doping/undoping process in the Ab/PPy 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 literature⁸ showed better defined semi-circles at DNA/PPy biosensors than that in Figure 6.2. This indicated that the Ab-Ag binding in the conductive polymer has a stronger effect on the doping/undoping process. However, it is difficult to analyze the impedance results with Randle equivalent circuit for R_{ct} 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 an additional reporter, $\text{Fe}(\text{CN})_6^{-4/-3}$ in their impedance measurements^{7, 15, 156} to detect IgG⁷, liposomes/biotin labeled target antibody and DNA^{15, 156} for the enhanced amplification of the Ab-Ag binding sensing processes. The mechanism is clear since $\text{Fe}(\text{CN})_6^{-4/-3}$ was used as reporters to probe the precipitation of labeled large molecules on electrodes, which could retard the electron transfer between the $\text{Fe}(\text{CN})_6^{-4/-3}$ redox pair to increase R_{ct} after Ab-Ag binding, which are not label-less or reporterless. Apparently, results obtained with label-less or reporterless impedance detection should have the new data analysis method, which will be discussed later.

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

AC impedances were measured using the anti-AZT/PPy 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 Figure 6.3(a) and the impedance results obtained with two electrodes in the solutions containing 0 and 100 ng/ml AZT are illustrated in Figure 6.3(b). In Figure 6.3, 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 Figure 6.3(a). Furthermore, the two electrodes in Figure 6.3(b) are supposed to have the same impedance before Ab-Ag interaction, but they show great variation in impedances. Deviations of the apparent surface area of the 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 Figure 6.3 (b), where instability of the Ab/PPy film was also exhibited because impedance change was observed before and after the incubation in AZT solution. This may be attributed to the variation of the deposited PPy film features since the electrochemical deposition process is greatly influenced by the chemical and physical properties of the electrode surface. It was unlikely that different electrodes have identical deposited PPy films with the same surface properties, even under well-controlled conditions 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 label-less 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 figures. 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 $\Delta R_N = (R_2 - R_1)/R_1$. 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 ($\Delta g/g_0$) used in literature⁷ where $\Delta g = g - g_0$; g_0 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 6.3 Impedance results using anti-AZT/PPy 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 multi-concentration 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 PPy films for eliminating or reducing the variation of bulk resistances caused by different Ab/PPy films. Furthermore, since there were no well-defined semicircles in the measured impedance for the Randle model, it was essential to analyze R_{ct} with other approaches. During data analysis, it was found 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 a few Hz to hundreds of Hz. The most significant part of the impedance at the frequency is equal to R_{ct} since R_{ct} is proportional to the concentrations of Ag. A software was designed to directly analyze impedance data for R_{ct} 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 6.3) were processed and it was found that the frequency band for the R_{ct} was around 50 – 60 Hz. Figure 6.4 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 a similar level to those of the best immunoassay methods that have been reported¹⁵⁹. Different concentrations of BAP, gp120, leptin, SA and biotin were detected with this label-less 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 label-less

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.

Figure 6.4 Relationship of Normalized Dimensionless Resistance Change, ΔR_N vs. AZT concentration.

6.3.3 Specificity of impedance label-less detection of proteins

Anti-AZT, anti-SA, anti-lepton, anti-BAP and anti-gp120 were used to prepare different Ab/PPy film electrodes for immunosensors using the method described in our previous work¹⁵⁷. Each of these Ab/PPy immunosensors was placed in different PBS solutions containing 25 ng/ml of different antigens to validate the specificity of the label-less impedance immunosensors. The experimental results in Figure 6.5 show the crossover reaction rates of each Ab/PPy immunosensor against different antigens.

Figure 6.5 Specificity of different Ab-PPy protein sensors. Concentration of all tested target antigens was 25 ng/ml in PBS.

Anti-AZT, anti-SA, anti-lepton, and anti-gp120 based on PPy immunosensors demonstrate good specificity against other antigens. However, the anti-BAP/PPy sensor had a 60% crossover rate with lepton in comparison with its paired BAP Ab-Ag binding. We used the ELISA method to determine the crossover reaction rate between anti-BAP and lepton. We found ~61% crossover reaction rate of anti-BAP against lepton. The crossover reaction rate measured from the label-less electrochemical immunosensor was very much 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 technique¹⁶⁰. Thus, the crossover reaction rate of anti-BAP with lepton occurred at the BAP/PPy immunosensor was not due to failure of the impedance label-less detection technique.

6.3.4 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 PPy film without antibody was deposited on the two gold electrodes as negative controls. The other two gold microelectrodes were employed for anti-gp120/PPy 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 6.6, 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_N from both the negative control and the anti-gp120/PPy electrodes were reduced. The reduction of ΔR_N with PBS washing indicated there were nonspecific bindings that occurred during

incubation in serum. However, the signal reduction of the negative controls with PBS washing was much more significant than that of the positive responses, indicating that the nonspecific binding on the PPy electrodes was much stronger than that of anti-gp120/PPy electrodes. This suggests that wash is a necessary step to significantly improve the measurement of the S/N ratio to enhance high sensitivity and specificity.

Figure 6.6 Nonspecific binding effect on Single to noise ratio for both anti-gp120/PPy film and plain PPy film electrodes.

6.4 Conclusions

A simple and sensitive impedance label-less detection based on an electrochemical immunosensor and its data processing method was investigated. One-step polymerization was used to impregnate antibody into the PPy film on glassy carbon electrodes for producing electrochemical immunosensors. The experimental results showed that the label-less impedance detection method could be used to detect different antibody-antigen interactions, of which the theory is that the impedance measurement can be used to sensitively detect the Faraday resistance for the doping/dedoping redox reaction of PPy. The Ab-Ag interaction in the PPy film can block the doping positions to retard doping/dedoping process of PPy and thus increase the charge transfer resistance. Based on this theory, it is clearly that using a smaller probe molecule such Ag to detect a larger target molecule such as Ab, which is normally much larger than Ag should have higher sensitivity. In fact, a

“sandwich” detection scheme, in which a second Ab is used to further bind the complexed Ab-Ag pair, can further significantly improve the signal to noise ratio (S/N). A 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/PPy films, leading to an S/N ratio that showed 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 the improvement of the 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 and 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 label-less impedance detection technology, a biochip could be developed with different antibody-attached probes to detect different antigens for high throughput analysis. In addition, the PBS wash demonstrated the improvement of the 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 the 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 PPy molecules. High efficient covalent method could be the solution, which is currently being studied in the author’s lab.

Chapter 7 PPa-Based Amperometric Immunosensor

7.1 Introduction

Immunosensing, a combination of specific immunoreactions with sensitive optical or electrochemical transduction, has attracted much attention since the 1970s¹⁶¹⁻¹⁶⁴. Owing to the adaptability of electrochemical sensing to miniaturization, considerable research efforts have focused on electrochemical arrayed immunosensors and biochips with high sensitivity and specificity. One of the key steps in the construction of electrochemical immunosensors is to select a pertinent method for probe immobilization. The most widely used method is the bead-based immobilization technique where probes are physically adsorbed or covalently bound to the surface of polystyrene microbeads with a magnetic iron core^{165, 166}. Although high sensitivity (femtomoles) has been reported,¹⁶⁷ this approach cannot provide reproducible and addressable deposition of relevant immunoreagents with controlled spatial resolution, thus limiting its application in microarray and biochips. Another alternative method is to employ an electropolymerized conducting polymer as a matrix to immobilize immunoreagents. After the pioneering work of Foulds and Lowe,¹⁶⁸ the immobilization of biomolecules such as enzymes, DNA, antibodies, and even whole cells in conducting polymers has been widely studied to fabricate biosensors, including immunosensors. Compared to the bead based method, the electrosynthesis of conducting polymers allows for precise control of probe immobilization on surfaces regardless of their size and geometry⁶⁶. Since the polymerization occurs on the electrode surface, the probes are essentially entrapped in proximity to the electrode. This feature is of particular importance toward the development of sensing microelectrodes and microelectrode arrays to shorten the response time and alleviate interference from the bulk solution. Furthermore, the amount of immobilized probes can be easily controlled either by changing their concentration or by adjusting the thickness of the polymer matrix through the electrode potential, electropolymerization

time, or both. Among all of the conducting polymers studied to date, PPy can be considered as one of the most attractive materials due to its excellent conductivity, stability, and biocompatibility, even in a neutral pH medium.^{169, 170} At present, most PPy based immunosensors are performed with impedance or potential step methods by measuring the changes in capacitance or resistance induced in antigen-antibody binding events, offering real-time and label-free measurement.^{48, 66, 77, 155, 171, 172} However, there are few reports on PPy-based amperometric immunosensors using a labeled enzyme to catalyze a substrate into a detectable electroactive product. The lack of widespread applications of PPy for amperometric immunosensing can be attributed to the poor permeability of PPy that hinders the diffusion of the electroactive product in and out of the polymer film.⁶⁶ This paper describes the construction of a new amperometric immunosensor using electrosynthesized PPa as immobilization matrix. Probes can be attached to the abundant carboxyl groups on the polymer surface or entrapped in the polymer network, leading to fast and reliable amperometric responses. In our studies, mouse IgG is selected as a model analyte and its detection is realized in the presence of p-aminophenyl phosphate (PAPP) using the sandwich mode with rat anti-mouse IgG and sheep anti-mouse IgG (conjugated to alkaline phosphatase) as the primary and second antibodies, respectively (Scheme 7.1). The performance of immunosensing with respect to detection sensitivity and reliability is presented and discussed in detail.

Scheme 7.1. Schematic of the amperometric enzyme immunosensor based on the PPa film. The electroactive product, p-aminophenol (PaP), was converted from the enzymatic conversion of PAPP by alkaline phosphatase (ALP): (a) entrapment mode; (b) covalent bonding mode using EDC as the cross-linker.

7.2 Preparation and characterization

7.2.1 PAPP synthesis

PAPP was synthesized as described in the literature.¹⁷³ In brief, 1 mL of *p*-nitrophenyl phosphate (PNPP; Sigma, St. Louis, MO) and 10 mL of 0.05 M H₂SO₄ were added to the cathode chamber, separated in the electrolytic cell by a Nafion membrane (Aldrich), where Pt foils were used as the cathode and anode electrodes. Electrosynthesis was carried out using the galvanostatic mode at 8 mA/cm² for 5 h, and the resulting solution was adjusted to pH 9.0 with sodium hydroxide.

7.2.2 Carboxylic group activation by EDC

EDC reacts with a carboxyl group first and forms an amine-reactive intermediate,

an O-acylisourea. The intermediate is unstable in aqueous solutions and is therefore not useful in two-step conjugation procedures without stabilization of the intermediate using N-hydroxysuccinimide. Failure to react with an amine will result in hydrolysis of the intermediate, regeneration of the carboxyl and release of an N-substituted urea. A side reaction is the formation of an N-acylurea, which is usually restricted to carboxyls located in the hydrophobic regions of proteins.

Scheme 7.2 EDC reaction with carboxylic acid and primary amine-containing molecules.

7.2.3 Electrochemical characterization

Glassy carbon (GC) electrodes were polished with 0.3 μm alumina powder, rinsed with deionized water, and then polished with 0.05 μm powder. After polishing, the electrodes were ultrasonically cleaned for 10 min in deionized water and finally soaked in acetone for 5 min, followed by vigorously washing with deionized water. Cyclic voltammograms of the polished electrodes were measured in 0.05M $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ to confirm that the potential difference between cathodic and anodic peaks was not more than 65 mV (the theoretical value is 59 mV).

Electrochemical impedance spectroscopy (EIS) was used to characterize PPa film formation, probe immobilization, and antigen-antibody interaction using an Autolab

potentiostat/ galvanostat (PGSTAT30) equipped with a frequency response analyzer module. All impedance data were recorded at an open circuit voltage with an amplitude of 10 mV over a frequency range of 1 Hz-1 MHz in 0.1 M PBS solution containing 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$.

The sandwich immunoassay was performed by dropping 150 μL of PAPP solution to cover the modified immunosensor. Differential pulse voltammetry (DPV) was used to detect amperometric responses because of its high signal-to-noise (S/N) ratio. In the DPV measurement, the pulse amplitude, pulse width, sampling width, and pulse period were 50 mV and 0.06, 0.02, and 0.2 s, respectively, and the potential range was -0.4 to +0.3 V versus Ag/AgCl.

7.2.4 FT-IR Characterization.

To identify the main functional groups on the surface of electropolymerized PPa films, attenuated total reflection Fourier transform infrared (ATR-FT-IR) spectroscopy was performed by using the Nicolet Magna IR 560 ESP spectrophotometer (Thermo Nicolet, Madison, WI) incorporated with a “Golden Gate” ATR module (Graseby Specac, Smyrna, GA). The IR data show that the strongest peak appears at 1730 cm^{-1} , which can be assigned to CdO in carboxyl groups, suggesting the abundance of carboxyl groups in the PPa film.

7.2.5 Immunosensor Construction.

Two methods were used for probe immobilization. For covalent bonding, Pp monomers were first electropolymerized on the GC electrode to form a PPa film by the galvanostatic method at 0.1 mA for 500 s. The coated electrode was washed three times with 0.1M PBS and dried under a gentle nitrogen stream. The electrode was then immersed in 150 μL of acetonitrile (ACN) containing 1.5 wt % EDC for 1.5 h at room temperature. After careful rinsing with ACN, the electrode was exposed to a solution containing 100 $\mu\text{g/mL}$ sheep anti-mouse IgG for 2 h, followed by washing and 1% BSA treatment for 2 h to alleviate nonspecific protein adsorption. The resulting

electrode was incubated with different concentrations of mouse IgG (0.1 ng/mL to 1 μ g/mL) for 2 h followed by washing with PBS. Thereafter, 100 μ L of 20 μ g/mL rat anti-mouse IgG (H+L) conjugated alkaline phosphatase (ALP) was dropped onto the electrode surface and incubated for 2 h. Finally, all the modified electrodes were rinsed with PBS before electrochemical measurement. In the entrapment method, 0.3 M PPa was mixed with 100 μ g/mL sheep anti-mouse IgG and then electrochemically polymerized to form a protein-doped PPa film. Incubation of antigen and binding of the second antibody were similar to that described above.

7.3 Results and discussion

7.3.1 Electrochemical Behavior of the PPy and PPa Films.

Figure 7.1 compares the cyclic voltammograms (-0.2 to +0.65 V vs. Ag/AgCl) of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ obtained on the PPy- and PPa-coated GC electrodes in 0.1 M PBS at pH 7.4. The film thickness was controlled by the charge passing during the electropolymerization step. Replacement of HN in pyrrole by the propylic acid group ($\text{CH}_2\text{CH}_2\text{COOH}$) resulted in different electrochemical behavior. During the growth of the PPy film, the redox peak current of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ declined sharply whereas the peak potential separation between anodic and cathodic waves increased drastically. Indeed, the 3-mC-thick PPy film did not display defined redox waves for $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$, indicating both the deactivation of PPy and the poor permeability of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ through the polymer film. Since most of the electroactive products such as H_2O_2 converted from the enzyme catalytic reaction exhibit higher positive oxidation potentials compared to the PPy film, the continual detection of these electroactive species will keep the polymer in an oxidized state for a long time and cause the loss of polymer conductivity, namely, deactivation.^{169, 174-178} Even the oxidation potential of electroactive products such as PaP is close to that of PPy; the electrooxidation of PPy (doping) in a broad potential range will increase the background current and thus decreases the S/N ratio. In addition, the dense structure and hydrophobic property of the PPy film after polymerization restrict the permeation

of electroactive species to the electrode surface. Without reconditioning (overoxidation),¹⁷⁴ the signal response of the PPy-coated electrode is very weak and undetectable, particularly at low analyte concentrations. In contrast, the redox waves of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ (curves a and b in Figure 7.1B) were almost identical even if the PPa film thickness increased from 0 to 10 mC. The peak current and potential separation were only slightly changed compared to the bare GC electrode (Figure 7.1B, curve a). For the 30-mC film thickness, the redox peaks (curve d in Figure 7.1B) were still observable but decreased gradually with the growth of the film thickness. This behavior was reasoned to result from the deactivation of PPa or the retarded diffusion of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ through the polymer film. To decipher this behavior, cyclic voltammograms of PPa and PPy measured in the PBS buffer were used to estimate their conductivity.

Figure 7.1 Cyclic voltammograms of 50 mM $\text{Fe(CN)}_6^{3-}/\text{Fe(CN)}_6^{4-}$ on polymer-coated GC electrodes with the scan range of -0.2 to +0.65 V vs. Ag/AgCl. (A) PPy electropolymerized with the charge of 0 (a), 1 (b), 2 (c), and 3 mC (d). (B) PPa electropolymerized with the charge of 0 (a), 10 (b), 20 (c), and 30 mC (d). Scan rate: 50 mV/s.

A pair of redox peaks in the PPa polymer was observed at 0.0 and -0.2 V versus Ag/AgCl (Figure 7.2, curve b), implying that the PPa film might also possess a doping and undoping process like PPy (Figure 7.2, curve a). However, the peak current of PPa was much lower compared with that of PPy, indicative of poor conductivity of the PPa film. Besides, it was found that the electropolymerization

potential of PPa was 2.3 V versus Ag/AgCl, compared to 0.82 V for PPy under the same conditions. In other words, the deposition of PPa film required higher overpotential than the level required for the PPy film. On this basis, PPa film could not be subjected to the loss of conductivity (or, namely, deactivation) even if held at high positive potential for a long period. Therefore, the decay in the redox peaks (Figure 7.2B) was mainly caused by the limited diffusion of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ into a thick PPa membrane. The same experiment was also carried out with PaP, the enzyme-catalytic product of PAPP, as an indicator. Similarly, that is, the PPy film drastically hindered the permeation of PaP while the PPa film appeared much more permeable. The CV curves of PaP and $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ recorded on PPa-coated electrodes both exhibited the increasing peak currents with the growth of cycles, which proved the mass-transfer process of PaP and $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ diffusion through the polymer film. However, the time required for PaP to reach its maximum peak current was much shorter compared to that for $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$, indicating a faster diffusion rate of PaP. A rationale behind such behavior could be explained as the electrostatic repulsion between the PPa film (negative charge) and $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. As a consequence, the permeation of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ was reduced. Obviously, the fast diffusion rate of PaP in the PPa film would lead to the reduced response time of the immunosensor.

Figure 7.2 (A) Comparison of polymer conductivity between PPy (curve a) and PPa (curve b) in 0.1 M PBS solution (pH 7.4) using cyclic voltammetry; (B, inset) amplified CV curve of PPa. Scan rate, 50 mV/s.

7.3.2 Impedimetric immunosensing based on PPa

PPa provides abundant carboxyl groups that enhance the film hydrophilicity and enable probe immobilization via covalent bonding. Electrochemical impedance spectra were used for monitoring the surface dynamics of the polymer, antigen and antibody interaction. $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ solution was used as the indicator to exhibit the faradic impedance change in the experiment. Nyquist plot was employed here and it includes the semicircles at high frequencies and follows straight lines at low frequencies. The semicircles portion corresponds to the charge-transfer-limited process with its diameter equal to the charge transfer resistance. The straight line was formed by the diffusion control of the reactant species. After anti-goat IgG immobilization on the PPa film, the semi-circle of the impedance increases drastically as shown in plot b. After the incubation of anti-goat IgG and goat IgG, the semicircle reaches its maximum. Both anti-goat IgG and goat IgG immobilization will cover the PPa film and thus retard the charge transfer process. The enlarged semicircles show the trend of the surface change of PPa film.

Figure 7.3 Impedance spectra of 10 mC PPa(a), PPa/anti-goat IgG(b) and PPa/anti-goat IgG/goat IgG.(c) in 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$, respectively.

The PPa, antibody and antigen interaction were also studied by impedance spectra in 0.01 M PBS buffer. Though the PPa has very weak electrochemical activity in comparison with PPy bearing no carboxyl group, the semicircle of the faradic impedance can still be observed in the impedance spectra. The same dynamics of the impedance spectra as the one in $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ can be observed in PBS. The semicircle in the PBS comes from the doping/dedoping process of PPa. The diameter of the semicircle in PBS is much larger than the one in $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. It is obvious that the charge transfer resistance of doping/dedoping process is much larger than that of the redox of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. However, the interaction of antibody, antigen and PPa can still be identified via the change of semicircle. The coverage of protein will retard the doping/dedoping process remarkably.

Figure 7.4 Impedance spectra of 10 mM PPA (a), PPA/anti-goat IgG (b) and PPA/anti-goat IgG/goat IgG (c) in 0.01 M PBS, respectively.

The electrochemical impedance change was monitored in 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ during the PPA film modification. The bonding dynamics of PPA and anti-goat IgG, and further anti-goat IgG and goat IgG result in the change in charge transfer resistance because of the surface coverage of protein, which will retard the indicator ions getting through the PPA film. The impedance spectra were recorded hourly. In Figure 7.5, it can be seen that both the charge transfer resistances became stable after 2 hours. The charge transfer resistance of anti-goat IgG and goat IgG bonding reached the maximum after 2 hours and then began to decrease slightly. This light decrease of the charge transfer resistance may be caused by the denaturation of the antibody and antigen in the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$, thus dissociation of the Ab-Ag. In comparison, the binding of anti-goat IgG and goat IgG is not affected because of the sturdy covalent binding between the carboxyl group and the amino group.

Figure 7.5 The charge transfer resistance change of PPa/anti-goat IgG (a) and PPa/anti-goat IgG/goat IgG (b) with time in 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$.

The charge transfer resistance of the PPa with or without protein will be varied by the quantity of the PPa. The quantity of the deposited PPa will affect both the redox species getting through the film and the protein immobilization capability due to the quantity of the carboxyl groups which will change the impedance effectively. The PPa quantity was varied from 10 to 30 mC and impedance spectra were recorded for each quantity with anti-goat IgG immobilization and antibody antigen binding. Figure 7.6 shows the normalized charge transfer resistance changes with the variation of the PPa quantity. The normalized R_{ct} increases with the PPa quantity gradually at the initial and reaches the maximum at 20 mC PPa. The increased PPa quantity will retard the redox species approaching the electrode surface but at the same time increase the immobilized antibody which will enhance the signal of impedance. The latter dominated the charge transfer resistance change before the PPa quantity less than 20 mC. After 20 mC, the normalized charge transfer resistance is much more depressed because the increased immobilized protein on PPa film can not compensate on the hindrance of large amount PPa to redox species.

Figure 7.6 The effect of PPA film quantity on charge transfer resistance change in 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ solution.

Figure 7.7 shows the calibration plot obtained in 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ solution. A linear relationship was observed over the range of 10 pg/mL to 10 $\mu\text{g/mL}$ with a detection limit of 10 pg/mL. This result illustrates PPA can be successfully used as matrix to immobilize probe protein for impedimetric detection.

Figure 7.7 Calibration curve of goat IgG in the range of 10 to 10^7 pg/mL^{-1} in 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ solution.

7.3.3 Structural Characteristics of the PPa Film.

Under positive potential and with long exposure, PPy gradually loses its conductivity due to the destruction of long-range conjugation in the polymer.¹⁶⁹ Thus, amperometric responses from electroactive species in the solution cannot be detected on the deactivated PPy surface. In the case of PPa with poor conductivity, the signal response was always observed regardless of the duration in which the polymer was kept at a positive potential. This result could only be explained by the difference in the polymer structures. In our experiment, AFM was used to probe the PPa film. The AFM micrographs of the bare GC electrode (Figure 7.8a,b) displayed some scrapping traces, caused by 0.05- μm alumina powder during electrode cleaning and polishing. When the electrode was covered with the PPa film, the AFM micrograph illustrated a porous structure without any appearance of scrapping traces (Figure 7.8c,d). The aperture size was mainly distributed over 0.2-0.5 μm , which was sufficient for the permeation of ions. The formation of the porous structure was not totally unexpected since charges could not efficiently transfer through the PPa film. Hence, a long-chain polymer could not be synthesized in the absence of free radicals, indicating a self-limiting process. From Figure 7.8c,d, the PPa film thickness was estimated to be 100 nm after charging with 0.1 mA for 500 s. For comparison, the AFM images of the PPy film prepared under the same condition were measured (Figure 7.8e,f). Obviously, the structure of PPy was very compact without any observable pores on the polymer surface. As a result, ions such as $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ and PaP cannot penetrate this film. Nevertheless, PPy could also become porous after overoxidation at a very high positive potential, resulting in detectable signals.^{152, 179} However, the activity of the immobilized biomolecules might be affected at high anodic potential when a single electropolarization step was used to entrap such biomolecules.

Figure 7.8 AFM images of the bare and coated GC electrodes. (a) and (b) Bare electrode surface after polishing with 0.05 μm alumina powder. Scan sizes are 50 and 10 μm . (c) and (d) PPa-coated electrode surface with the charge of 50 mC. (e) and (f) PPy-coated electrode surface with the charge of 50 mC.

7.3.4 Characterization of Polymer Coating and Protein Immobilization/Binding Events on the Electrode Surface.

Abundant carboxyl groups on the PPA polymer surface enhance the film hydrophilicity and enable probe immobilization via covalent bonding. To attest the attachment of antigen and antibody onto the electrode surface, EIS was used for monitoring the dynamics of the polymer and Ag-Ab interaction.¹⁸⁰ Due to the absence of an internal redox couple during the polymerization and protein immobilization/binding events, $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ was added to the solution as the indicator to acquire faradic impedance spectra. Note that the attachment of polymer and proteins to the electrode surface retards the interfacial charge transfer of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ and thus increases the electron-transfer resistance.¹⁸¹ Figure 7.9 illustrates the EIS profile, in which the Nyquist plot is shown with the real part (Z'') on the X -axis and the imaginary part (Z') on the Y -axis. The impedance spectra in Figure 7.9 were measured on a GC electrode before and after the polymer coating, anti-mouse IgG immobilization, anti-mouse IgG/mouse IgG interactions, and the attachment of ALP conjugated rat anti-mouse IgG, respectively. The spectra illustrated well defined semicircles at high frequencies followed by straight lines at low frequencies. The semicircle portion corresponded to the electron-transfer-limited process with its diameter equal to the electron-transfer resistance (R_{ct}). The straight line was formed by the diffusion control of the reactant species. The lowest electron-transfer resistance on the bare electrode (Figure 7.9a) indicated the fastest electron-transfer rate between the bare electrode and the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ pair. With the PPA film coated surface (Figure 7.9b), the electron-transfer resistance increased dramatically, owing to the high electrode coverage by the PPA layer with poor conductivity. After probe antibody immobilization, antigen-antibody binding, and the second antibody attachment (Figure 7.9e), Faraday resistances R_{ct} increased accordingly, indicating the successful construction of the immunosensor. However, the increase in R_{ct} was not as drastic as that caused by the PPA film since the electrode surface was only subjected to low coverage of proteins. Such results confirmed the

applicability of PPa in electrochemical impedance immunosensing.

Figure 7.9 EIS of the PPa-coated GC electrode in 0.1 M PBS solution containing 10 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. (a) Bare electrode; (b) PPa-coated electrode; (c) modified with sheep anti-mouse IgG; (d) combined with mouse IgG; (e) coated with sandwich complexes of ALP-conjugated rat anti-mouse IgG.

7.3.5 Performance of the Immunosensor.

Mouse IgG was used as the target protein in the electrochemical sandwich immunoassay experiments, involving immobilization of the primary antibody (Ab, sheep anti-mouse IgG), capture of the analyte (Ag, mouse IgG), association of alkaline phosphatase conjugated rat anti-mouse IgG, and finally incubation in a PAPP solution.^{141, 162, 182} For the detection, ALP converted PAPP to *p*-aminophenol (PaP), which was electrooxidized to *p*-quinone imine at 0 V versus Ag/AgCl (eqs 1 and 2).

Scheme 7.3 the reactions in the detection.

In our study, the enzyme substrate PAPP was synthesized as described previously (Experimental Section). In brief, the nitro group in PNPP was electroreduced or

chemically reduced by the hydrogen produced during electrosynthesis to form an amino group (eq 3).¹⁷³

Differential pulse voltammetry (DPV) can be used to achieve sensitive responses from the immunosensor.¹⁸³ Figure 7.10 shows the differential pulse voltammograms of PaP measured with an interval time of 130 s. The concentration of target protein, mouse IgG, was 100 ng/mL, and the probe was covalently immobilized to the PPa film with EDC as the cross-linker. As shown in Figure 7.10, virtually no peak current was detected right after the addition of PAPP. With prolonged time, the peak current increased, reflecting the increase in the PaP concentration. The peak current-time linear relationship (Figure 7.10B) indicated zero-order kinetics for the enzymatic reaction. The rate increase of the PaP concentration (the slope of I vs t) was proportional to the amount of enzyme conjugated to the second antibody, i.e., proportional to the amount of antigen to be detected. According to the DPV theory, the slope of I versus t is a function of the electrode surface area and the amount of enzyme. To normalize the surface area, all electrodes involved in the experiment were measured in the same PaP solution and their relative areas were obtained by comparing the obtained peak currents. Figure 7.11A shows the calibration plot obtained with the covalent bonding mode; i.e., probes were covalently attached to the PPa surface. Measurements were performed in triplicate using three different immunosensors. A linear relationship was observed over the range of 100 pg/mL-1 μ g/mL, illustrating the successful development of the PPa-based amperometric immunosensor. Besides covalent bonding, PPa could also serve as a matrix to directly entrap probes, thus simplifying the preparation of the immunosensors. The porous structure of the PPa film enables the target proteins to interact with the probes encapsulated in the polymer matrix. Figure 7.11B presents the corresponding calibration data, illustrating that the detection limit of the entrapment mode was an order higher than that of the covalent bonding mode. This could be ascribed to the higher concentration of probes immobilized in the PPa film by the covalent bonding method compared with the entrapment procedure. The measurements were conducted

in human serum at room temperature. Immunoglobulins (Ig) have five different Ig classes (IgG, IgM, IgD, IgA, IgE) based on differences in the constant region of the heavy chain. Frequent doses of antigen produced by infectious diseases usually lead to a strong IgG response in human serum. Thus, the immunosensor was validated in human serum for its specificity and sensitivity. The detection limit was observed in the same order range compared with the data obtained in PBS (Figure 7.11C), validating the high specificity of the proposed immunosensor. To investigate the stability of immunosensors, the electrode was stored in PBS buffer at 4 °C and examined every 24 h, repetitively. The amperometric responses retained 85% of the initial activity after 24 h and 71% after 4 days. The results showed that the proposed immunosensor possesses satisfactory stability although biosensors are often designed as disposable devices in clinical practices.

Figure 7.10 (A) DPV curves of PaP at various times with the increasing rate of 130 s by use of the covalent bonding mode; (B, inset) peak current dependence on time.

Figure 7.11 Calibration curves of mouse IgG using covalent bonding mode in PBS buffer (A), entrapment mode in PBS buffer (B), and covalent bonding mode in human serum (C). Detection at each concentration was repeated three times, and the results were averaged.

7.3.6 Further Optimization of the Immunosensor.

A series of experiments were performed to investigate the pH effect on the signal response and nonspecific interaction. The experimental results indicated that the background current decreased with an increase of the solution pH. Figure 7.12 compares the amperometric signals of the negative control in PBS, pH 7.4, and the carbonate bicarbonate buffer, pH 9.6. In brief, the background current in the carbonate-bicarbonate buffer was suppressed significantly, showing reduced nonspecific adsorption of ALP on the PPa film in a solution at high pH. One of the possible reasons could be ascribed to the stronger electrostatic interaction between ALP and the PPa film at lower pH, resulting in a higher background. In PBS, pH 7.4, ALP acquires a positive charge (pI 8.17) while PPa carries a negative charge due to the presence of carboxylate anions. Thus, the amount of ALP nonspecifically adsorbed on the PPa-coated electrode surface at the negative control was not negligible even after the blocker treatment. However, above pH 8.17, ALP carries a negative charge. Apparently, this could significantly reduce the nonspecific binding of ALP on the PPa film caused by the electrostatic interaction. As a result of the optimization, 20 pg/mL mouse IgG could be detected using the covalent bonding mode in the carbonate-bicarbonate buffer, which was 5 times lower than the detecting limit in PBS. It should be noted that the background current of the covalent bonding mode (Figure 7.12b,d) was much smaller than that of the entrapment bonding mode (Figure 7.12a,c). Such results could be attributed to the lower amount of nonspecific bound ALP on the PPa film by the covalent bonding mode.

Figure 7.12 Amperometric responses at negative control in various buffer solutions. (a) Entrapment mode in PBS, pH 7.4; (b) covalent bonding mode in PBS; (c) entrapment mode in carbonate-bicarbonate, pH 9.6; (d) covalent bonding mode in carbonate-bicarbonate, pH 9.6.

7.4 Conclusions

In brief, the novelty of the immunosensor described herein consists of the porous structure and hydrophilic property of PPa film, which enable the detection of amperometric responses from the electroactive product during the course of the immunoassay. The abundance of carboxyl groups on the polymer surface permits immunoreagent immobilization via covalent bonding, leading to high detection sensitivity compared with the traditional entrapment method. Following investigation on the use of PPa together with electrochemical sensing, significant improvements with regard to sensitivity can be achieved. With high reproducibility, the technique could have great potential toward the development of miniaturized protein biochips for clinical, forensics, environmental, and pharmaceutical applications.

Chapter 8 PPy/PPa/Au Nanocomposite Based Impedimetric Immunosensor

8.1 Instruction

Impedimetric immunosensors have attracted increasing interest because of their potential advantages over amperometric and potentiometric ones with respect to direct and label-less detection, short assay time, convenient multiplexing sensing and simple operation^{77, 184, 185}. However, they have often suffered from low detection limit. An 100 pM detection limit was achieved with a new sensing method¹⁸⁶, which is not good enough to meet the requirements for most clinical diagnostic applications. Thus, impedimetric sensors face great challenges for significantly improving their sensitivity and detection limit.

Conductive polymers such as polyaniline, polythiophene, and PPy in electrochemical sensors have been extensively investigated⁵⁻¹⁷, of which PPy exhibits various advantages of good biocompatibility, simple synthesis, and easy immobilization of various biomolecules¹⁸⁻²². Previously, Li et al^{8, 77} have demonstrated impedimetric DNA and protein sensors based on pristine PPy thin films. However, the sensors applications are limited from their instability and insufficient sensitivity. The low sensitivity can be attributed to the poor permeability of PPy, which hinders the diffusion of the reactant/product in and out of the polymer film⁶⁶. PPa, a derivative of Py, can form a highly porous and hydrophilic thin film and is used to build an amperometric immunosensor with good sensitivity¹⁸⁷, but it has much higher resistivity than PPy and apparently is not good enough to be directly used for a highly sensitive impedimetric immunosensor due to a low S/N (signal to noise) ratio from its intrinsic high impedance.

In recent years, PPy based nanocomposites have been studied due to their unique properties inherited from individual components. PPy based nanocomposites, particularly PPy-metal nanocomposites are studied to enhance their physical properties²³. PPy/Au nanocomposite is developed to improve the PPy electroactivity

and stability⁸⁹. In PPy/Au nanocomposite, Au nanoparticles can function as a large counterion to retard PPy degradation¹²⁴ and can also enhance the hydrophilicity due to its surface hydrophilic groups. This may make PPy/Au more suitable in the application of biomedical devices¹²⁵, especially in electrochemical sensor application with improvement from both conductivity and stability.

One of the great challenges in construction of a highly sensitive impedimetric immunosensor is to explore an efficient probe immobilization method. Entrapment is a simple and convenient way to immobilize biomolecules in PPy film, but the immobilization solution is required to be mild for avoiding biomolecule deactivation. Furthermore, the random orientation of immobilized biomolecules may lose biological activity due to the spatial hindrance which is very hard to be controlled by entrapment approach. Covalent binding is a better strategy for biomolecules immobilization, in which optimal conditions could possibly be used for better probe immobilization while increasing the probe density through increasing the linker density in the PPy film. Pa is a homemade compound with COOH groups for covalent immobilization of biomolecules. Considering the individual advantages of PPy, PPa and Au, the nanostructured PPy/PPa/Au composite is designed in this work and may provide unique properties for a highly sensitive and stable impedimetric immunosensor, in which the sensing film could benefit from good conductivity of PPy, hydrophilicity and covalent binding ability of PPa and stability and conductivity of Au nanoparticles.

IL-5 is an interleukin produced by CD4⁺ T cells and a 115 amino acid long TH2 cytokine in human²⁶, which stimulates B cell growth and increases immunoglobulin secretion^{27, 28}. IL-5 has been associated with several allergic diseases including allergic rhinitis and asthma.¹⁸⁸ Strong evidence indicates that IL-5 plays an important role in allergic asthma pathology¹⁸⁹. Therefore, quantitative detection of IL-5 has great significance for disease pathology study, clinic diagnosis and pharmaceutical research. Enzyme-linked immunosorbent spot (ELISPOT) and enzyme-linked immunosorbent assay (ELISA)^{31, 190-192} are conventional methods for

immunological detection. Although some detection kits are commercially available, the assay is time-consuming, labor-intensive, operation skill demanding, and also requires expensive optical devices. In comparison to ELISA and ELISPOT, an impedimetric IL-5 immunosensor can apparently render a fast, inexpensive, convenient and portable detection technique.

In this work, we investigate PPy/PPa/Au nanocomposite based human IL-5 label-less impedimetric immunosensor, which includes synthesis of the PPy/PPa/Au nanocomposite and its optimization for high electroactivity and stability. RAMAN, cyclic voltammetry (CV), AFM and AC impedance were used to characterize or/and optimize the nanocomposite. The IL-5 sensor calibration curves were obtained in PBS and PBS + 1% human serum solutions to evaluate their sensitivity and specificity.

8.2 Preparation and Characterization

8.2.1 Raman Characterization

Raman spectroscopy was performed with Renishaw Micro Raman spectrometer by using 632.8 nm lines of the He-Ne laser as the excitation source over the range of 400-2000 cm^{-1} .

8.2.2 Characterization of Carboxylic Group Density.

The density of the immobilized proteins is very critical parameter for evaluation of the immobilization efficiency. The absolute density of immobilized proteins can be measured via the radioactive labeling method, but we did not conducted due to the limited experimental conditions. However, the density of the immobilized protein can be indirectly expressed by density of the carboxylic group on the surface that immobilized the proteins. The density of the carboxyl in the films was quantified by using Toluidine Blue O (TBO) method¹⁹³. The polymer coated electrodes were soaked in an aqueous solution of 5×10^{-4} M TBO, adjusted to pH 10 with NaOH. The formation of ionic complexes between the COOH group and cationic dye was

carried out for 5 hours at room temperature. The unreacted dye was removed by rinsing with pH 10 NaOH solution. Desorption of the dye was conducted in 50 wt% acetic acid solution. The quantity of COOH group was measured by the absorbance of dye at 633 nm with a HITACHI U-2800 double beam system.

8.2.3 Immunosensor Fabrication.

A typical protocol to fabricate the PPy/PPa/Au nanocomposite immunosensor is presented here and detail optimization work is discussed later in this report. The precursor solution for polymerization contained 0.26 M pyrrole, 0.065 M pyrrole propylic acid, 0.01M PBS and 0.15 mM Au nanoparticles. The PPy/PPa/Au nanocomposite film was electrochemically deposited by applying 1.5 mAcm^{-2} constant current for 300 s. The coated electrode was washed three times with 0.01M PBS and dried under a gentle nitrogen stream. The electrode was then immersed in acetonitrile containing 1.5 wt% EDC for 1.5 h at room temperature. After carefully rinsing with acetonitrile, the electrode was exposed to a solution containing $100 \mu\text{g/mL}$ IL5 overnight, followed by washing in PBS to eliminate nonspecific protein adsorption. The fabrication process is summarized in Scheme 1.

Scheme 8.1. Schematic of the immobilization of the protein probe via the activation of carboxylic functional groups (activated by EDC).

8.3 Results and discussion

8.3.1 Effect of Incorporated Au Nanoparticle on Electrochemical Behavior of PPy Based Nanocomposite Film.

The effect of incorporated Au nanoparticles on the electrochemical behavior of the produced PPy/PPa/Au nanocomposite film was investigated. The electropolymerization of the PPy/PPa/Au nanocomposite film was carried out by using the parameters described in the experimental section, except for the variables discussed here. The $E-t$ (E : deposition potential, t : deposition time) in Figure 8.1a shows that the deposition potential of PPy/PPa is always higher than that of PPy/PPa/Au during the whole deposition process at a constant current, indicating that the PPy/PPa has higher polarization potential and slower deposition rate. Particularly, the PPy/PPa deposition potential sharply increases to 0.94 V, compared to 0.85 V for the PPy/PPa/Au, demonstrating an even faster deposition rate at the beginning. Clearly, the lower polarization potential and faster deposition rate comes from the incorporation of Au nanoparticles into the composite. In our previous work¹⁹⁴, the AFM *in situ* study of PPy/Au deposition process reveals that the Au nanoparticle has stronger affinity with the electrode surface than Py for facilitating the nucleation and growth process. CVs of the PPy/PPa and PPy/PPa/Au films, which have the same amount of electrode material because of their same synthesized electricity (Figure 8.1b), exhibit two well-defined doping/dedoping redox peaks. The peak current of the PPy/PPa/Au film is significantly higher than that of PPy/PPa and much less peak potential separation for the PPy/PPa/Au (0.056 V) than that of PPy/PPa (0.105V). The higher peak current and less peak potential separation of the PPy/PPa/Au film can be ascribed to high electrochemical reactive surface area and greater reaction reversibility, respectively, and then indicate it has much higher electroactivity than that of PPy/PPa. CVs obtained with different scan rates (Figure 8.1c) illustrate symmetric features and the linear relationship of peak current vs. the scan rate indicates surface electrode reactions with fast charge transfer rates.

Figure 8.1. The electrochemical behavior of PPy/PPa/Au nanocomposite. a, The galvanostatic deposition of the PPy/PPa film (solid line) and the PPy/PPa/Au nanoparticle film (dashed line) on the glassy carbon electrode. b, Cyclic voltammograms of PPy/PPa and PPy/PPa/Au nanoparticle film in 0.01 M PBS obtained with a scan rate of 100 mV/s. c, Cyclic voltammograms of the PPy/PPa/Au film in 0.01 M PBS with different scan rates: 20 (A), 40 (B), 60 (C), 80 (D), 100 (E), 120 (F), 140 (G) mV/s. d, The dependence of the oxidation peak current on the scan rate.

8.3.2 Morphology of PPy/PPa and PPy/PPa/Au Films.

Besides the electrochemical behavior changes, the morphology of PPy/PPa/Au composite film also exhibits great differences from PPy/PPa. AFM images reveal that the PPy/PPa/Au (Figure 8.2b) film exhibits a significantly rougher and more porous structure compared with the PPy/PPa film (Figure 8.2a). The larger nanoparticles of PPy/PPa/Au are due to the influence of Au nanoparticles during the

polymer growth¹⁹⁴. The mean roughness data of the PPy/PPa and PPy/PPa/Au films are 3.9 and 13.7 nm, respectively, calculated from the AFM images obtained by sampling the whole measured area, indicating that the PPy/PPa/Au film has a much larger specific surface area. This may be one of the important factors for the film to have higher electroactivity than PPy/PPa. Au nanoparticles produced in our work have a protection layer from aggregations, with negative charged carbonyl groups that can covalently bind with monomer Py or Pa and can be the nuclei for electrochemically PPy and PPa deposition, resulting in polymerization from both Au nanoparticles and Py/Pa monomers. In the deposition of the PPy/PPa/Au film from both Au nanoparticles and Py/Pa nuclei, the Au nanoparticles should have faster nucleation and growth rate than Py and Pa monomers, since an Au nanoparticle can attach to a number of monomer Py or Pa molecules, resulting in larger polymerized particles than that nucleated from Py and Pa. This is also demonstrated in our previous work studied by *in situ* AFM measurements¹⁹⁴. This is why a less uniform, rougher and porous morphology of the PPy/PPa/Au film is observed in Figure 8.2.

Figure 8.2. AFM characterization of a, the PPy/PPa film and b, the PPy/PPa/Au nanocomposite (height scale=60 nm).

8.3.3 Effect of Incorporated Au Nanoparticles on Stability and Conductivity of PPy Based Composite Film.

The electrochemical stability comparison between PPy/PPa/Au and PPy/PPa films was carried out by monitoring impedance changes of these film-based electrodes in 0.01 M PBS at room temperature for a relatively long period. The impedance result is often modeled by an electronic equivalent circuit for the solution resistance, R_s , the Warburg impedance, Z_w resulted from the diffusion of ions in a bulk electrolyte, the double layer capacitance, C_{dl} , and R_{ct} for the electrochemical reaction. The increased R_{ct} for the doping/dedoping process of a PPy thin film for a long time measurement is used to represent the degradation of the polymer in its electroactivity. In our previous work⁷⁷, a normalized resistance, ΔR_{ct} is introduced to eliminate the variation of PPy films when multiple detections with different electrodes. In a study of the stability of the PPy based composite film, the ΔR concept is used and can be expressed as

$$\Delta R_{ct} = (R_{ct2} - R_{ct1}) / R_{ct1} \quad (1)$$

where definition of R_{ct1} and R_{ct2} are different from that used to monitor the protein bindings, and here represent the impedance measured at the beginning and at a time to check the stability, respectively. The change of ΔR_{ct} of PPy/PPa/Au film in 2.5 hours is less than 10% while PPy/PPa film increased by 23% in the same time frame as shown in Figure 8.3a. The change of the charge transfer resistance should have resulted from the degradation of the composite film and could be ascribed to damages of the positively charged backbone of the polymer by the nucleophilic attack from the solution species¹²³. Apparently, the incorporation of Au nanoparticles significantly enhances the stability of the PPy composite. It is known that a large counterion doping in a conductive polymer can effectively inhibit the nucleophilic attack⁸⁹ for better stability. In PPy/PPa/Au film, it is likely that the negatively charged Au nanoparticles can function as a very large counterion doped into the polymer matrix for the improved stability.

Figure 8.3b shows the current-voltage (I-V) curves of PPy/PPa and PPy/PPa/Au films under dry condition. The linear relationship of I vs. V indicates a good resistor nature of both films and the slopes can represent their conductivity. The conductivity calculated from the I-V curves are 2.11×10^{-5} and 5.52×10^{-5} S for PPy/PPa and PPy/PPa/Au films, respectively, demonstrating that PPy/PPa/Au film has 2.6 times better conductivity than PPy/PPa film. To study the mechanism of the improvement of the conductivity, the Raman spectra of these films were measured as shown in Figure 8.3c. The peak located in the range of $1513.5\text{--}1646.8\text{ cm}^{-1}$ represents the C=C backbone stretching of PPy^{132, 133}, which is related to the conjugating length of the polymer chain and the conductivity of the polymer¹³⁴. The peak position of the C=C backbone shifts from 1596.0 to 1589.7 cm^{-1} in the PPy/PPa/Au film in comparison to the PPy/PPa film, indicating that the conjugation is shortened in PPy/PPa/Au composite, thus resulting in higher conductivity. The peak in the range of $1020\text{--}1080\text{ cm}^{-1}$ can be assigned to the C-H in-plane deformation. The Raman peak intensity can characterize the size of the anion dopant, of which the larger size can result in higher conductivity¹⁰⁰. The increased peak intensity at 986.7 cm^{-1} for the PPy/PPa/Au film shown in Figure 8.3c can be assigned to the larger “anion” dopant, and the negatively charged Au nanoparticles can also contribute to improved conductivity¹⁰⁰. The Raman spectrum results clearly explain the improvement mechanism of the conductivity improvement. Thus, incorporation of Au nanoparticles into the composite not only improves the stability but also the conductivity of the conductive polymer. The conductive polymer composite film with significantly improved stability and conductivity is expected to have much better sensitivity and reliability in electrochemical sensor applications, particularly in an impedimetric sensor application.

Figure 8.3. Comparison of PPy/PPa and PPy/PPa/Au nanocomposite. a, Electrochemical stability comparison of the PPy/PPa/Au and the PPy/PPa film in 0.01 M PBS at room temperature. b, Current voltage (I-V) curves of PPy/PPa/Au and PPy/PPa. c, Raman spectra comparison of the PPy/PPa and the PPy/PPa/Au nanocomposites.

8.3.4 Effect of Deposition Current Density Magnitude on Electroactivity of PPy/PPa/Au Film.

PPy/PPa/Au composite deposition was carried out by using constant current to give better electroactivity.¹⁰¹ In order to optimize the film for the impedimetric sensor, different current densities were applied while passing the same electric charge density of 0.429 C cm^{-2} by controlling the deposition time. The electroactivity of the resulted films was examined by CV in 0.01 M PBS and the results are shown in Figure 8.4, of which the two pairs of redox peaks have been studied for their electrochemical reactions in our previous investigation⁸⁹, that the films deposited with high current density of 1.77 mA cm^{-2} and 1.06 mA cm^{-2} have lower redox peaks. The loss of the electroactivity is likely due to overoxidation at such deposition current densities¹²³. The composite films produced with current densities of 0.71 mA cm^{-2} , 0.53 mA cm^{-2} and 0.35 mA cm^{-2} show the best performance in both peak current and reversibility. The films deposited with low current densities of 0.18 mA cm^{-2} , 0.11 mA cm^{-2} and 0.035 mA cm^{-2} deposition show high redox peaks, but have worse reversibility than that deposited with current density of 0.71 mA cm^{-2} , 0.53 mA cm^{-2} and 0.35 mA cm^{-2} . The low deposition current density forms more dense morphology (data not shown), since a low current density causes a slow deposition rate that allows better alignment of the polymer chains for less porosity of the film¹⁹⁵. The worsened reversibility is very possibly caused by a sluggish doping/dedoping process in the films. To compromise electroactivity and reaction surface area from the porosity, 0.35 mA cm^{-2} 300s was chosen as the constant current density to synthesize the PPy/PPa/Au for further optimization works.

Figure 8.4. CV of PPy/PPa/Au films in 0.01 M PBS which were synthesized in 30 mC quantity by applying different current: 1.77, 1.06, 0.71, 0.53, 0.35, 0.18, 0.11, 0.035 mAcm⁻². (scan rate: 50 mV/s, vs. Ag/AgCl. reference electrode).

8.3.5 Effect of Py to Pa Ratio on Sensitivity of PPy/PPa/Au Film Based Impedimetric Immunosensor.

Because of the nature of PPy and PPa, of which PPy is conductive but hydrophobic, while PPa is less conductive but very hydrophilic, it is critical to optimize the ratio of Py to Pa to have a composite film with good conductivity and hydrophilicity for a sensitive impedimetric immunosensor. The ratio of Py to Pa in the film can be tailored by adjustment of the monomer ratio of Py to Pa. The electrochemical activities of the films produced with different deposition solutions containing different Py to Pa ratio were evaluated by CV as shown in Figure 8.5a. The results illustrate that film (A), (B), (C) and (D) produced with a Py to Pa ratio lower than 1 have very weak redox peaks due to their poor electrochemical activities, clearly caused by the high percentage of PPa in the composite. However, when the ratio is over 1, the film (F), (G), (H) and (I) show better electrochemical activities. The poor electroactivity of the composite films formed by a low [Py]:[Pa] ratio is attributed to their high percentages of the poorly conductive PPa⁴. The poorly conductive PPa can also result in high deposition potential⁴. Thus a high ratio of PA to Py in the deposition solution could leads to overoxidation of PPy to produce a

degraded composite film with poor electroactivity. In addition, a reasonable amount of PPa is needed to provide a high concentration of COOH groups for excellent covalent bindings. According to the experimental results, the [Py]:[Pa] ratio should be higher than 2.3. Results in Figure 8.5b shows the density of COOH group in films synthesized with three different [Py]:[Pa] ratios with or without the incorporation of Au nanoparticles. In all three ratios, the films incorporated with Au nanoparticles increase their carboxylic group density as high as 124.4%, 230.3% and 312.5% of that of the films without Au nanoparticles, respectively. Apparently, the higher density of COOH groups in PPy/PPa/Au film comes from the anti-aggregation layer on the surface of Au nanoparticles as discussed above.

Figure 8.5. Optimal [Py] to [Pa] ratio for PPy/PPa/Au nanocomposite films. a, CV of the PPy/PPa/Au films in 0.01 M PBS which were electrochemically deposited in the solution containing different [Py]:[Pa] ratio: (A) [Py]:[Pa]=1:9, (B) [Py]:[Pa]=1:4,

(C) [Py]:[Pa]=3:7, (D) [Py]:[Pa]=2:3, (E) [Py]:[Pa]=1:1, (F) [Py]:[Pa]=3:2, (G) [Py]:[Pa]=7:3, (H) [Py]:[Pa]=4:1 and (I) [Py]:[Pa]=9:1. b, COOH group density comparison of different PPy/PPa films with or without the existence of Au nanoparticles: in precursor solutions (A) [Py]:[Pa]=7:3 with Au nanoparticles, (B) [Py]:[Pa]=7:3 without Au nanoparticles, (C) [Py]:[Pa]=8:2 with Au nanoparticles, (D) [Py]:[Pa]=8:2 without Au nanoparticles, (E) [Py]:[Pa]=9:1 with Au nanoparticles, (F) [Py]:[Pa]=9:1 without Au nanoparticles.

Anti-rabbit IgG Impedimetric immunosensor was fabricated from rabbit IgG-immobilized PPy/PPa/Au films to explore the optimal [Py]:[Pa] ratio in the deposition solution. Figure 8.6 shows the immunosensing responses to different [Py]:[Pa] ratios, which were used to synthesize the composite films for the sensors. The measurements were conducted in 100 ng/mL anti-rabbit IgG + 0.01 M PBS. The result shows that the immunosensor response increases with the increase of the ratio until reaching its maximum value at 4:1 and then sharply dropping to 9:1. This result indicates that when the [Py]:[Pa] ratio is smaller than 4:1, the film has enough COOH groups for probe molecule bindings and the sensitivity of the impedimetric sensor is mainly dependent on the film electroactivity. However, when the ratio continues to increase to hit at a ration of 9:1, the film may not have enough COOH functional group for the covalent binding of the probe molecule, resulting in a significant reduction of the sensitivity. Impedimetric immunosensors made with different probe protein-immobilized PPy/PPa/Au composite films were fabricated to investigate their optimal [Py]:[Pa] ratio of the deposition solution, and almost the same optimal ratio value was obtained. Therefore, the optimal [Py]:[Pa] ratio of the deposition solution is determined as 4:1.

Figure 8.6. Relation of [Py]:[Pa] ratio and the response of the immunosensor based on these ratio in 0.01M PBS.

8.3.6 Optimal Incubation Time for Immunosensor Detection.

ΔR_{ct} was monitored in 100 ng/mL anti-rabbit IgG + 0.01M PBS and anti-IL5 IgG + 0.01M PBS respectively with 10 minutes' interval to investigate the optimal incubation time for the immunosensor. As shown in Figure 8.7, in the first 30 minutes, the ΔR_{ct} increases rapidly and after 30 minutes this trend is slowed down and plateaus to show the saturation. Therefore, we choose 60 minutes as the optimal incubation time for our immunosensor.

Figure 8.7. Optimal incubation time for the immunosensor. a. immunosensor using rabbit IgG as probe in 100 ng/mL anti-rabbit IgG solution. b. immunosensor using IL5 as probe in 100 ng/mL anti-IL5 IgG solution.

8.3.7 IL-5 Impedimetric Immunosensor.

The immunosensor performance for anti-IL5 IgG detection was tested in both 0.01M PBS and 1% human serum solution. Figure 8.8a shows the calibration curves of ΔR_{ct} vs. different concentrations of anti-IL5 IgG in PBS with a linear relationship over a dynamic range of 4 orders of magnitude and 10 fg/mL detection limit. This is a very sensitive electrochemical sensor, which can satisfy most of the critical application requirements. This significant improvement in sensitivity can be attributed to the improved stability, good conductivity, high hydrophilicity, high porosity and efficient covalent probe-immobilization ability of the PPy/PPa/Au

composite sensing layer, which are clearly inherited from the individual components of the composites, i.e. PPy, PPa and Au nanoparticles. The measurements were also conducted in 1% human serum to further evaluate the immunosensor anti-interference performance. Immunoglobulin (Ig) has five different Ig classes (IgG, IgM, IgD, IgA and IgE) based on differences in the constant region of the heavy chain. Frequent doses of antigen produced by infectious diseases usually lead to a strong IgG response in human serum. Thus, the immunosensor was validated in human serum for its specificity and sensitivity, and the result is shown in Figure 8.8b. The detection limit of the sensor in human serum is 1 pg/mL in a dynamic range between 1 pg/mL to 1 ng/mL, demonstrating a good sensitivity and specificity. Selectivity of this immunosensor was investigated into 1 ng/mL anti-goat IgG, anti-rabbit IgG and anti-IL-5 IgG + 1% human serum solutions, respectively. Figure 8.8c shows that the immunosensor gives the significant response to specific analyte in comparison with nonspecific ones, indicating good selectivity. The detection limit in serum of the sensor has 1 pg/ml, which is equal or better than the commercially available ELISA test kits (from a few pg to 50 pg/ml. Because of its great advantage of simplicity, low cost, fast assay time, and ability to be used in an electronic and even portable device, the impedimetric immunosensor reported in this work should have great broad applications in clinical diagnosis and environmental monitoring. However, although the device is sensitive, the detection limit in serum is significantly reduced from 10 fg/ml to 1 pg/ml. This is because in detection in serum, a much larger noise is observed to significantly reduce the S/N ratio of the sensor.

Figure 8.8. Performance of hIL-5 Sensor. a, Anti-IL-5 antibody concentrations: 1000 pg/mL, 100 pg/mL, 10 pg/mL, 1 pg/mL and 0 pg/mL. b, Selectivity test of the sensor

in 1% human serum solution containing 1 ng/mL anti-rabbit antibody, anti-goat antibody and anti-IL-5 antibody, respectively.

8.4 Conclusions

In brief, our study shows PPy/PPa/Au composite owns much better stability, porosity, hydrophilicity, and efficient probe immobilization capability in comparison with pristine PPy material that we used previously in the construction of an immunosensor. Excellent sensitivity and selectivity of this composite based sensor have been demonstrated via anti-IL5 IgG detection. The significant improvement of sensitivity is ascribed to the joint contributions from the individual components in the composite, in which PPa enhances the hydrophilicity and provides covalent probe immobilization linkers, PPy promotes the conductivity and electroactivity, and Au nanoparticles render good conductivity, high stability and covalent binding linkers as well. Most importantly, because Au nanoparticle play an important role in the nucleation and growth of the composite film, a highly porous nanostructured composite is formed to have high reaction surface for a high sensitivity. This work demonstrates a universal approach to fabricate highly sensitive sensors through nanocomposite, which not only can inherit individual advantages of the composed components, but also can benefit its synergy effect such as the porosity structure of PPy/PPa/Au nanoparticles. This nanocomposite based immunosensor has great potential to construct sensing devices for clinical, pharmaceutical, environmental and food safety applications.

Chapter 9 PPy/PPa/Au Nanocomposite Based Regenerable Impedimetric Immunosensor

9.1 Introduction

Immunosensors have been the mainstream in clinical diagnosis and are used extensively in pharmaceutical research, environmental monitoring and food safety applications due to their superior specificity, sensitivity and convenience. Various immunosensors based on a broad range of receptors and transducers have been reported^{3, 154, 196-202}. The main drawback of these immunosensors is their high cost because they have to be abandoned after detection. Thus, a renewable sensing component of an immunosensor is highly desirable²⁰³⁻²⁰⁶. However, the construction of a regenerable immunosensor is hard to achieve, because the immunological reactions characterized by low dissociation rate constants and multivalent interactions of high avidity are often virtually irreversible²⁰⁷. Such interactions require extremely harsh regeneration conditions that eventually lower the antibody binding capacity²⁰⁸. Affinity-based immobilization to overcome the regeneration problems encountered in the regeneration process can be solved by protein G and protein A. Protein A from *Staphylococcus aureus* can selectively bind the Fc region of a wide range of IgG²⁰⁹⁻²¹¹. Protein G from group G *Streptococci* has a more versatile binding capability of Fc region of IgG with high affinity²¹²⁻²¹⁴ with no interference to the antigen binding sites. These features make them very attractive as affinity-capture supports for immunological sensing applications²¹⁵. Furthermore, the IgG immobilized on protein G can have a better orientation than those randomly immobilized on a surface which can result in the loss of biological activity²¹⁶⁻²¹⁸. In a random orientation of IgG, its F_{ab} fragments may be hidden, which result in the antibody-antigen binding being hindered.

Immunosensors using protein G or protein A as affinity ligand based surface by amperometry²¹⁵, chemiluminescence^{219, 220} or surface plasmon resonance²⁰⁸

transduction have been reported. These immunosensors need enzyme labeling or expensive optical component to carry out detection. Electrochemical immunoassay has been growing rapidly and making significant advances because its measurements are simpler, less expensive, and even more precise over optical detection methods in some cases. Our earlier works have demonstrated impedance label-less immunosensors^{8, 77}, which impedance transducers are based on thin PPy film. Although a PPy thin film can be simply deposited on different electrode substrates by various electrochemical methods¹⁰¹, its hydrophobic nature leads in aqueous media to non-permeable films for poor electroactivity¹⁸⁷. This problem can be solved by a copolymer of Pa and Py, of which Pa can provides both the covalent binding capability and improved hydrophilicity^{187, 221}. Incorporation of Au nanoparticles into the thin PPy film can further improve the hydrophilicity, stability and electroactivity^{89, 194}. However, there is no reported PPy based regenerable electrochemical immunosensor to date.

Leptin is a 16 kDa protein hormone that plays an important role in regulating food intake and body composition, as discovered by Friedman et al.^{222, 223} The binding of leptin to the ventromedial nucleus gives the brain the signals of sensation of satiety. In healthy individuals, a decrease in leptin increases neuropeptide production in the hypothalamus, which in turn increases appetite and decreases energy expenditure²²⁴. The obesity which is caused by homozygous mutations for the leptin gene can be treated by administration of recombinant human leptin²²⁵. The detection of leptin has been reported by various researchers via either immunocytochemical²²⁶⁻²²⁹ method or radioimmunoassay²³⁰⁻²³². However, these detection methods are expensive, tedious and time consuming. Thus, there is a need to develop a convenient, fast and low cost immunosensor for detection of leptin.

Various protein G-based detection techniques have been used in quartz crystal microbalance²³³, surface plasmon resonance²³⁴ and ellipsometry²¹⁸. However, protein G associated electrochemical impedance detection has not been reported. In this study, we report the impedimetric label-less immunosensors based on affinity

protein G immobilized PPy/PPa/Au film, which can combine the advantages from PPy/PPa and PPy/Au nanocomposites^{89, 221}. The regeneration process of protein G and anti-leptin IgG binding is investigated by electrochemical impedance spectroscopy. The optimal regeneration time of IgG on protein G/PPy/PPa/Au nanocomposite film is studied by optical and impedance measurements. The leptin detections are carried out in both 0.01 M PBS and 1% human serum to confirm the reproducibility of the regenerable immunosensor.

9.2 Preparation and characterization

9.2.1. Immunosensor fabrication

The deposition solution contained 0.26 M Py, 0.065 M Pa, 1×PBS solution and 0.15 mM Au nanoparticles. The PPy/PPa/Au nanocomposite film was electrochemically deposited by applying 1.5 mAcm⁻² constant current for 300 s. The electrolyte solution was deaerated by purging with nitrogen before use. The synthesis conditions were optimized and reported by us¹⁰¹.

The attachment of protein G onto the copolymer film was performed according to a previously reported procedure²³⁵, in which the electrodes were soaked in acetonitrile containing 1.5 % EDC for 1.5 hours, followed by the addition of 8 µL 1 mg/mL protein G solution onto the electrode surfaces and then kept at room temperature for 12 hours. Finally, the modified electrodes were thoroughly washed with 0.01M PBS solution to remove unattached protein G on the electrode surface.

9.2.2 Immunosensor regeneration

Anti-leptin IgG immobilization on protein G layer was conducted by immersing the modified electrodes in 1.6 mg/mL anti-leptin solution for 30 minutes. After the formation of protein G – IgG binding, the modified electrodes were rinsed with 1×PBS thoroughly to remove the non-specific bound anti-leptin IgG. To elute the IgG, the electrodes were rinsed in 0.1 M glycine buffer (pH = 2.7) for 70 seconds. After elution, the electrodes were washed in 0.01M PBS solution for

re-equilibration.

9.3 Results and discussion

9.3.1. Impedance measurement of the probe regeneration process

The preparation scheme of the regenerable protein G/PPy/PPa/Au based immunosensor is shown in Figure 9.1. The electrochemical doping/dedoping process of a conductive polymer is used for simple and label-less detections of target DNA and proteins by impedance spectroscopy in our previous work^{8, 77}. Based on the same principle, the fabricated immunosensor as shown in Figure 9.1 is employed to conduct protein regenerable detection.

Figure 9.1. Preparation scheme of the regenerable PPy based immunosensor.

Impedance measurements were used to verify the regeneration process of the

immunosensor by glycine buffer, and the results are shown as Nyquist plots (Z_{im} vs Z_{re}) in Figure 9.2, of which curve a, c and e are obtained with the protein G-immobilized layer only after the recycle operation (curve a is the first measurement), while curve b, d and f are measured after binding of anti-streptavidin IgG and protein G, respectively. A typical Nyquist plot can show a semicircle region lying on the axis over a high frequency range followed by a straight line, of which the radius of the semicircle portion corresponds to the charge transfer resistance, R_{ct} whereas the linear part is characteristic of the lower frequencies range, representing the diffusion limited electrochemical process. The impedance result is often modeled by a electronic equivalent for the solution resistance, R_s , the Warburg impedance, Z_w resulted from the diffusion of ions in a bulk electrolyte, the double layer capacitance, C_{dl} , and R_{ct} for the electrochemical reaction⁸. The increased R_{ct} for the doping/dedoping process of a PPy thin film after antigen binding with the immobilized antibody in the film can be used to detect the target molecules as antigen in a sample¹⁵⁷. Figure 9.2 illustrates the typical impedance characteristics of an electrochemical reaction, and clearly shows that R_{ct} -the radius of the semicircle increases after immobilization of protein G. However, the baselines (curve a, c and e) after removal of the bound antibody by regeneration are not the same. The variation is possibly ascribed to the film variation of the PPy/PPa/Au nanocomposite film during the regeneration although the stability of PPy/PPa/Au is largely improved by nanocomposing with Au nanoparticles⁸⁹. In our previous work⁷⁷, a normalized resistance, ΔR is introduced to eliminate the variation of PPy films when multiple detections with different electrodes are required. ΔR can be expressed as

$$\Delta R = (R_2 - R_1) / R_1 \quad (1)$$

where R_1 and R_2 are the impedance measured before and after the binding of a target biomolecule and the probe in the PPy film. The concept is used to process the impedance results from the regenerable immunosensor studied here, in which we used a normalized charge transfer resistance, $\Delta R_{ct} = (R_{ct2} - R_{ct1}) / R_{ct1}$ instead of directly measured R_{ct} from Nyquist plots and the result is shown in the inset of Figure 9.2,

demonstrating that protein G-immobilized PPy/PPa/Au nanocomposite film can keep almost the same baseline of R_{ct} after regenerations and can be used for a regenerable immunosensor.

Figure 9.2. Impedance change in regeneration process of protein G and anti-streptavidin binding, protein G layer only: a, c and e; affinity complex of anti-streptavidin IgG and protein G layer: b, d and f.

9.3.2 Efficient immobilization and removal of probe antibody

To determine efficient times for immobilization incubation and removal elution of the antibody probes, both impedance and optical measurements were conducted to the amount of HRP labeled anti-mouse IgG bound with protein G on the protein G/PPy/PPa/Au film before and after incubation or elution, respectively. The differences of normalized charge transfer resistance (ΔR_{ct}) and absorbance measured before and after immobilization incubation or removal elution of anti-IgG are further normalized to a same scale, i.e. both maximum normalized resistance and absorbance are equal to 1 for comparison for comparison, as shown in Figure 9.3. For assessment of the optimal incubation time, the protein G/PPy/PPa/Au film electrode was placed in 0.01 PBS for an impedance measurement as the baseline, and then immersed in 11 $\mu\text{g/mL}$ HRP labelled anti-mouse IgG solution for different

times, followed by rinsing electrodes with 0.01 M PBS to remove non-specific bound HRP labelled anti-mouse IgG. The impedance was measured after incubation as the indicator of immobilized antibody quantity. For optical measurements, the electrode was incubated for different times to immobilize the HRP labelled anti-mouse IgG, the same as used for the impedance measurement, and then 10 μ L TMB, which can be converted to a color indicator by HRP, was dropped onto the surface of the HRP labelled anti-mouse immobilized electrode, which was vertically fixed on a support to hold the TMB solution on its surface without loss, for 3 minute incubation in the dark. After incubation, 8 μ L of reacted TMB solution on the electrode surface was taken and transferred with pipettor to a microplate well, which contains 10 μ L 1M HCl to stabilize the color of reacted TMB, followed by an optical measurement with the microplate reader describe above. Both impedance and optical measurements were conducted for incubation times of 1, 3, 5, 10 minutes, and then at 10 minutes intervals until 70 minutes, to show a consecutive change. The results in Figure 9.3 show that the antibody and protein G binding is saturated after an incubation time of 40 minutes (99.3% binding) in the optical data. The impedance result is in agreement with that measured by the optical method, although it has a slight deviation. The result confirms the impedance measurement can be used to detect the protein bindings and indicates that 60 minutes is the optimal incubation time for interaction of antibody with protein G for the best immobilization of the impedimetric immunosensor.

Figure 9.3 Optimal incubation time for IgG immobilization on protein G/PPy/PPa/Au nanocomposite film.

The elution time required for IgG removal from the protein G/PPy/PPa/Au nanocomposite film was also studied by both impedance and optical measurements. The 0.1 M glycine buffer (pH=2.7) was used to disassociate IgG from protein G on the electrode because protein G temporarily loses its binding capability with IgG in a low pH environment²⁰⁸. The HRP labelled anti-mouse IgG immobilized electrode was immersed in glycine buffer for different times from 0 to 80 s, followed by rinsing with 0.01 M PBS to terminate the disassociation after each elution, and then impedance and optical measurements were taken by the same procedure described above. Figure 9.4 shows that disassociation rates with different elution times measured by both methods are almost the same, of which minor deviations possibly come from measurement variations, and 70 seconds elution time can achieve more than about 95% dissociation of the antibody and protein G bindings. The results in Figure 9.3 and 4 strongly demonstrate the reliability of the label-less impedimetric detection and consistency with optical measurements.

Figure 9.4. Optimal elution time for IgG removal from protein G/PPy/PPa/Au nanocomposite film.

9.3.3. Leptin detection of the immunosensor

Leptin was selected as the target antigen to test the performance of the regenerable immunosensor. Anti-leptin, an IgG was immobilized on the protein G/PPy/PPa/Au electrode as the probe to form the immunosensor. The immunosensor was placed in both 0.01 M PBS and 0.01 M PBS + 1% human serum solutions containing various concentrations of leptin for the impedance measurements, respectively. In Figure 9.5a, the calibration curves measured in 0.01 M PBS shows that the immunosensor has detection limit of 10 ng/ml and dynamic range of 10 ng/mL to 100 μ g/mL. Figure 9.5b shows the results obtained with the immunosensor in both initial and regenerable uses in 0.01 M PBS + 1% human serum, demonstrating that the immunosensor after the regeneration has the same detection limit of 10 ng/ml and dynamic range of 10 ng/mL to 100 μ g/mL, and the estimated concentration of the protein for leading to the saturation of the sensor signal is above 1 mg/mL. However, it is noted that in comparison to the calibration curve measured in 0.01 M PBS + 1% human serum, the immunosensor has lower sensitivity of 3.1 Ω / μ g/mL than that measured in solutions containing 0.01 M PBS, although it possesses the roughly

same detection limit and dynamic range. This is likely ascribed to the high baseline in the 1% human serum for a low signal to noise ratio (S/N).

Figure 9.5 Calibration curve of the immunosensor tested in 0.01M PBS (a), in 1% human serum and after one regeneration in 1% human serum (b).

9.4 Conclusions

Regenerable and label-less leptin immunosensor based on protein G/PPy/PPa/Au nanocomposite film was developed. Protein G was covalently immobilized on the PPy/PPa/Au nanocomposite film with EDC. Both impedance and optical measurement are employed to characterize the regenerable process and prove the feasibility of the regenerable impedimetric immunosensor by the consistency

between their measured results. The leptin impedimetric immunosensor exhibits a detection dynamic range of 10 – 100,000 ng/mL with 10 ng/mL detection limit in both 0.01 M PBS and 0.01 M PBS+1% serum solutions. However, due to the serum interference, the detection sensitivity of the immunosensor is slightly reduced in comparison to that in 0.01 M PBS. This immunosensor provides great potential to fabricate a regenerable label-less immunosensor with applications in environmental control and food analysis.

Chapter 10 General Conclusions and Future Work

10.1 General conclusions

In this dissertation, the advancement of PPy based electrochemical immunosensors in recent years has been reviewed thoroughly. The details including detection mechanisms, immunosensor construction and signal process have been presented and compared. Amperometric, potentiometric and conductometric transducers have been applied for immunosensor construction, respectively. Though the label-less detection on a wide range of immunological agents have been achieved by the immunosensors based on PPy, several drawbacks including hydrophobic nature and unsatisfied stability of pristine PPy in aqueous solution still restrain their performance and applications. To conquer these challenges, PPy, PPa and PPy based nanocomposite made with PPa and Au nanoparticles were investigated thoroughly and used to construct the different immunosensors.

To build more stable PPy for immunosensors, Au nanoparticles were incorporated into the PPy matrix to form PPy/Au nanocomposite film with a one step electrochemical deposition method. In order to deeply understand the role of Au nanoparticles in PPy/Au nanocomposite, *in situ* AFM measurement was used to characterize the nucleation and growth process of PPy/Au nanocomposite film in nanoscale. The data derived from the *in situ* AFM images associated with particle analysis is used to explore the deposition process of PPy/Au. It is discovered that negatively charged Au nanoparticles can be as nuclei for Py adsorption and polymerization and doped in PPy matrix as counterion, and thus give a higher nucleation and polymerization rate than Py alone at the initial polymerization stage. In addition, the PPy/Au nanocomposite film growth is decided by both Au nanoparticles and the Py nucleation processes. This makes the deposition kinetics of PPy/Au close to the theoretical instantaneous 3D nucleation model with slight deviations. Further TEM and SEM investigation showed that Au nanoparticles can

be incorporated into the PPy matrix with their own size unchanged while making the PPy film more porous and conductive. The result of Raman spectroscopy indicates that the C=C backbone in PPy/Au film was shortened to result in an enhanced conductivity. Furthermore, Au nanoparticles can act as large anions being doped into the PPy matrix to retard the PPy degradation process. Therefore, PPy/Au nanocomposite film shows much better stability than pristine PPy does. The contact angle measurement found that Au nanoparticles can increase the hydrophilicity of the resulted PPy/Au film due to the hydrophilic groups coated on Au nanoparticle surface. Furthermore, CV in $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ shows that PPy/Au has enlarged electroactive surface area due to Au nanoparticles incorporation.

First, a simple and sensitive impedimetric immunosensor based on PPy was successfully constructed by one-step polymerization with impregnate antibody into PPy film. The experimental results showed that the label-less impedance detection method could be used to detect different antibody-antigen interactions. A normalized dimensionless unit for impedance change was introduced to analyze the impedance data successfully. The method using resistance change is exploited to eliminate or reduce the variation of the electronic properties of Ab/PPy films, leading to significant S/N ratio 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 the S/N ratio. 10 pg/ml detection limit and superior specificity were demonstrated by the methods. Based on this label-less impedance detection technology, a biochip could be developed with different antibody-attached probes to detect different antigens for high throughput analysis.

Pa possesses one propylic acid group on its N position, and is homemade by us. The novelty of the immunosensor constructed with the porous structure and hydrophilic property of the PPa film, which enables the detection of amperometric responses from the electroactive product during the course of the immunoassay was reported. The abundance of carboxyl groups on the polymer surface permits immunoreagent immobilization via covalent bonding, leading to high detection

sensitivity compared with the traditional entrapment method. Following investigation on the use of PPa together with electrochemical sensing, significant improvements with regard to sensitivity can be achieved.

Our study shows that PPy/PPa/Au nanocomposite owns much better stability, porosity, hydrophilicity, and efficient probe immobilization capability in comparison with pristine PPy material that we used previously in the construction of an immunosensor. Excellent sensitivity and selectivity of this composite based sensor have been demonstrated via anti-IL5 IgG detection. The significant improvement of sensitivity is ascribed to the joint contributions from the individual components in the composite, in which PPa enhances the hydrophilicity and provides covalent probe immobilization linkers, PPy promotes the conductivity and electroactivity, and Au nanoparticles render good conductivity, high stability and covalent binding linkers as well. Most importantly, because Au nanoparticles play an important role in the nucleation and growth of the composite film, a highly porous nanostructured composite is formed to have high reaction surface for a high sensitivity. This work demonstrates a universal approach to fabricate highly sensitive sensors through nanocomposites, which not only can inherit individual advantages of the composed components, but also can benefit its synergy effect such as the porosity structure of PPy/PPa/Au nanoparticles.

Depending on our research on label-less impedimetric immunosensor based on PPy/PPa/Au nanocomposite film, one regenerable leptin immunosensor was developed successfully. Protein G was covalently immobilized on the PPy/PPa/Au nanocomposite film with EDC. Both impedance and optical measurement are employed to characterize the regenerable process and prove the feasibility of the regenerable impedimetric immunosensor by the consistency between their measured results. The leptin impedimetric immunosensor exhibits a detection dynamic range of 10 – 100,000 ng/mL with 10 ng/mL detection limit in both 0.01 M PBS and 0.01 M PBS + 1% serum solutions. However, due to the serum interference, the detection sensitivity of the immunosensor is slightly reduced in comparison to than in 0.01 M

PBS.

In brief, PPy, PPa and PPy/PPa/Au nanocomposite films based immunosensors can offer protein detections with high sensitivity and specificity. We have successfully demonstrated the detections on various important proteins in pg/mL or even fg/mL in diluted serum or PBS. Due to the easy and flexible polymerization of PPy, PPa and PPy/PPa/Au nanocomposite, these immunosensors can be promising to be constructed on printed electrodes or microchips with simultaneous detection of different analyte in high throughput. Our immunosensors have exhibited the potential to be produced in inexpensive, sensitive and portable sensing devices for practical applications. Exploration in this direction could lead to an important influence in immunosensor research which is expected to provide better sensing devices in the future.

10.2 Future work

Future study can be directed to several critical aspects for more deeply scientific insights and practical applications. The results in this work show that the charge transfer resistance of doping/dedoping process for PPy/PPa/Au increases after the Ab-Ag interaction and the linear relationship can be plotted for clinical diagnosis; however, the effects of type of probes, molecular size, steric effect and charge of the analyte on the detection sensitivity and specificity are still not clear. SPR can be integrated with impedance spectroscopy to conduct this investigation. For practical applications, miniaturization is extremely important. Protein chips, arrayed protein sensors for high throughput and inexpensive multiple analyte detection is another challenging work. By using screen printer, electrochemical multi-detection biochip with high throughput can be designed and fabricated on a plastic substrate for disposable and inexpensive sensing devices. Another issue we should pay attention is the interference from the real sample. To further improve the sensitivity, S/N ratio needs more improvement. Our work has showed that the diluted human serum has interference to the immunosensor response. For real sample such as blood, saliva and

urine, the interference may become more complex. Thus, the endeavor to alleviate the interference is worthy to study on both sample pretreatment and data process.

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Publication list

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