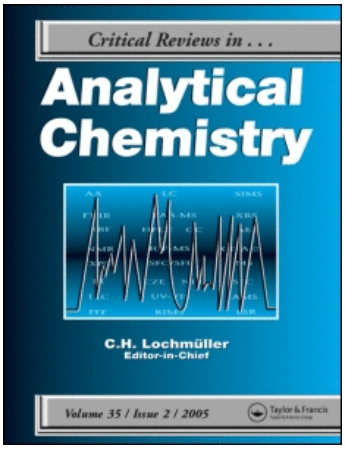

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Application of Polypyrrole for the Creation of Immunosensors

A. Ramanaviciene[1] *and A. Ramanavicius*[1,2]*

[1]Laboratory of Ecological Immunology, Institute of Immunology, Molètų pl., 2600, Vilnius, Lithuania;
[2]Department of Analytical and Environmental Chemistry, Vilnius University, 2009, Vilnius, Lithuania.

* e-mail: arman@bchi.lt

ABSTRACT: This review focuses on the use of conducting polymer (CP) films in electrochemical affinity sensors and emphasizes innovative designs and unique applications of immunosensors. The review covers some aspects in the application of polypyrrole (Ppy) for the creation of immunosensors. Polypyrrole film fabrication methods like solvent casting, adsorption, and electropolymerization are presented. The focus, is on electrochemically synthesized Ppy as very promising material for the formation of miniaturized electrochemical immunosensors. Polypyrrole films implemented in various capacities in amperometric, conductometric, and potentiometric immunosensor design are reviewed. The acceptance of immobilization and detection approaches used recently in affinity sensors with critical analysis applied in certain techniques is discussed. The biologically active components (BAC) used for the creation of polypyrrole-based immunosensors are described briefly. Some future trends in the development of polypyrrole-based immunosensors are predicted, as well as possible directions discussed.

KEY WORDS: conducting polymers, polypyrrole, immunosensors, immobilization, antibody.

I. INTRODUCTION

The quantitative analysis of selected components from complex biological and environmental samples traditionally has been achieved by the time-consuming and expensive combination of highly sophisticated chromatographic and spectroscopic techniques.[1] The requirement for the real-time monitoring of given analytes in medical, industrial, and environmental applications has meant that alternatives to bulky laboratory-based procedures had to be found, especially in the case of *in vivo* analysis.[2]

In recent years advances in the miniaturization and construction of electronic components and the fast response times offered by electrochemical techniques has created a demand for fast bio- and immuno-sensing information-collecting systems that can be easily integrated into technology, such as microprocessor-based electronics.[3] For these applications, biosensors, immunosensors, and biomimetic affinity sensors are required, as they are suitable for mass production. The biosensors and immunosensors are usually defined as sensing devices consisting of a biological recognition element in intimate contact with a suitable transducer, which is able to convert biological recognition reaction or eventually the biocatalytic process into a measurable electronic signal.[4] Immunosensors and affinity sensors based on molecularly imprinted polymers or high-affinity RNA aptamers are analytical devices that detect the binding of analyte by coupling immunochemical reaction to the modified surface of the device known as a transducer.[5] They are used mainly in areas where both high selectivity and high sensitivity are required.[6] Immunosensors have been the subject of increasing interest mainly because of their potential application as an alternative immunoassay technique in areas such as clinical diagnostics and environmental control.[7] The conversion of the binding event into a measurable signal in particular at low concentrations, the regenerability, and the reusability are, among other topics, major challenges in immunosensor development research.[8] Piezoelectric quartz crystal microbalance (QCM), surface

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scanning, like atomic force microscopy (AFM), and electrochemical transducers are used for the conversion of binding event into the analytical signal. As the detection limit of surface plasmon resonance (SPR), QCM, and atomic force microscopy is usually in the nanomolar range, many strategies have been developed for improvement, including, for SPR, the use of liposomes, latex beads, colloidal gold or hydrogels and plasma-polymerized films with stacked binding locations.

The effective combination of immunochemistry and electrochemistry in an analytical device could provide the basis of direct electrical detection of wide range of analytes with great sensitivity and specificity.[9] Direct detection immunosensors are the most attractive because they require no additional immunochemicals.[8] Antigen-antibody (Ag-Ab) complex formation in direct detection immunosensors can be detected electrochemically by alternating current, impedance and potential step methods. In these cases differences of capacitance and/or resistance are measured.[10] Because of simple miniaturization possibilities the electrochemical immunosensors are most suitable for the direct detection of analyte in the body of the patient investigated.

The major indispensable condition during the creation of immunosensors is that one of the able to bind reagents is immobilized, and at least one must be found in the sample.[11] It means that the immobilization of biologically sensitive compounds is one of main questions during the creation of immunosensors.[12] The conducting polymers can be considered effective materials for creation of immunosensing devices and especially for the immobilization of BAC.[13] The electrochemical modification of electrodes by conducting polymers doped with BAC included within polymeric backbone is a simple step that can be used for creation of immunosensors. On the other hand, CPs are capable of predetermined molecular interactions that can be modified *in situ* in a controlled fashion. They are also capable of transfering energy as electrochemical transducers.[14] During Ag-Ab interactions differences in capacitance and/or resistance arising in electrochemical system can be converted into electrical signals that are easily monitored.[15]

Some conducting polymers, such as polianiline, polytiophene, or polypyrrole (PPy), are biocompatible and, hence, cause minimal and reversible disturbance to the working environment and protect electrodes from fouling[16] and/or interfering electrochemically active materials.[17] Polypyrrole is used mostly in biosensors and immunosensors because of the best biocompatibility and the ease of immobilization of various biologically active compounds. Polypyrrole-based selective and stimulus-responsive biopolymers prove to be promising as new materials in immunosensors especially for biomedical application where direct detection of analyte is desirable.[18]

Another class of substances of great interest in the field of chemical sensor technology are molecularly imprinted polymers. These highly stable synthetic polymers possess molecular recognition properties due to cavities in the polymer matrix that are complementary to the analyte (ligand) both in shape and in positioning of functional groups.[19] Some of these polymers have shown very high selectivity and affinity constants fully comparable to naturally occurring recognition systems, such as antibodies, which makes them especially suitable for use in immunosensors. Polypyrrole imprinted by charged and neutral molecules has been applied in some affinity sensors.[20]

This article examines the usefulness of polypyrrole for creation of immunosensors and molecularly imprinted polymer based sensors.

II. DISCUSSION

A. Biological Recognition Elements of Immunosensor Construction

Almost all materials that are more or less affine to analyte can be used in immunosensors. In immunosensors based on conducting polymers can be applied: (1) antibodies/antigens;[21,22] (2) animal tissues;[23,24] (3) whole cells;[25-30] (4) DNA;[31-35] (5) molecular imprinting[36-37] (Ppy can be used itself as molecularly imprinted biomimetic material).

Antibodies are considered to be well-suited recognition elements for immunosensors. The high specificity and affinity of an antibody for its antigen allows a selective binding of the analyte (antigen), which is present in nano- to pico-molar range in the presence of hundreds of other substances, even if they exceed the analyte concentration by 2 to 3 orders of magnitude.[22]

At the time, antibodies can be generated against almost all analytes, even if the analyte is nonimmunogenic. Moreover, recombinant antibody technology has now been developed to a level that allows the expression of single chain fragments in *E. coli* in large quantities.[38]

Immunosensors for determination of nucleic acids. The detection of specific DNA fragments by hybridization with a complementary strand has gained considerable interest because of its importance to the early diagnosis of diseases.[6]

Molecularly imprinted conducting polymers. The stabilization of the biological response is currently a major problem, with almost every reported sensor exhibiting a gradual degradation in the electrical signal over time.[20] It is due to the instability of BAC used in the design of immunosensors. The resolution of this problem and the production of robust designs, vital for medical and environmental monitoring applications, can be based on creation of synthetic molecular recognition systems. It is the reason why the development of synthetic recognition systems is of great interest to workers in the field of sensor technology.

Molecular imprinting is a technology for the manufacture of synthetic polymers with predetermined molecular recognition properties.[36] The preparation of molecularly imprinted polymers (MIPs) requires polymerization around a print species using monomers that are selected for their capacity to form specific and definable interactions with the print species. Cavities are formed in the polymer matrix, which are images of the size and shape of the print molecules. Furthermore, chemical functionalities of the monomer residues become spatially positioned around the cavity in a pattern that is complementary to the chemical structure of the print molecule.[37] These imprints constitute a permanent memory of the print species and enable the imprinted polymer to rebind the print molecule from a mixture of closely related compounds selectively. Finally, the print molecules are removed by solvent extraction and the molecularly imprinted polymer is ready to be used. In some instances very high selectivities and affinity constants have been reported, fully comparable to naturally occurring recognition system such as antibodies.[36] Some of these synthetic polymers have been shown to be useful in sensor applications, exhibiting tolerance toward acid, base, high temperature, and organic phases.[37]

However, the majority of experimental results based on MIPs in real samples still exibit drawbacks with regard to affinity, cross-reactivity, and unspecific binding.

Other biologically active materials used for development of immunosensors. A huge number of different labels, including enzymes, redox compounds, cofactors, and liposomes, have been applied in electrochemical immunosensors.[39] Biotin and avidin as coupling able agents are applied in Ppy based biosensors as a tool for imobilization of BAC.[40]

No data about application of biomimetic RNA aptamers in Ppy based imunosensors were published. It looks that there is a great possibility for applying conducting polymers together with RNA aptamers.

B. Methods Used Mostly for the Fabrication of Polypyrrole Films

Conducting polymers are used widely as recognition elements in chemical sensors. They can be gas/vapor or ion sensitive. CP can be applied as immobilization matrix for BAC like biomolecules, cells, or polymer particles. Electrical 'wiring' of BAC is possible.[14] Among conducting polymers, PPy is one of the extensively studied electronic materials, and thus has received much attention because of various technological applications.

Polypyrrole is usually synthetisized by electrochemical and chemical oxidative polymrization techniques. Chemical polymerization occurs after the oxidation of pyrrole monomers and oligomers by oxidators to the cation-radicals that are recombining and forming polymeric structure of polypyrrole.[41]

Chemical methods are difficult to use for miniaturization, construction of sensor arrays, or the optimization of surface microenvironments.

Solvent casting is an extensively used procedure in the formation of CP films on electrodes. In this approach, a polymer film is formed by evaporation of a polymer solution (polypyrrole can be synthesized before chemically or electrochemically) placed on the electrode surface. The method is limited because it is difficult to control precisely as well as to obtain compact and uniform film.

Electropolymerization is a more modern and elegant method of polymeric film deposition. 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. Generally, polypyrrole films are formed by electrochemical anodic oxidation of a monomer and are insoluble, conducting or in some cases insulating polymer films that coat the electrode surface.[42] This is especially attractive, because the oxidation of monomer solution under the appropriate conditions gives results in a film deposited on the surface of the electrode and enables control of growth rate and film thickness. The electrochemical formation of CP films has found increasing interest in the development of bio- and immunosensors because they allow nonmanual reproducible formation of modified electrode surfaces with integrated biological recognition elements.[43] The use of conducting polymers for immobilizing BAC in sensor applications has the advantage, compared with conventional immobilization procedures, because the amount of deposited material can be readily controlled and the immobilizing matrix can conduct electricity allowing switch between conducting and isolating state.

Electrochemical film formation can be performed using potential cycling methods, fixed potential techniques, pulsed potential approaches, and galvanostatic techniques.

C. Immobilization Methods Based on Application of Conducting Polymers

Conducting polymers such as polypyrrole, polythiophene, and polyaniline are provided to be most useful molecular structures for these applications. A number of techniques to immobilize a BAC on the electrode surface are available, including adsorption, covalent attachment, cross-linking, and entrapment within polymeric chain.

Adsorption is the simplest way to immobilize BAC on the surface of electrode coated by polypyrrole. This electrode can be coated by polypyrrole electrochemically by using one of the mostly used electropolymerization methods.[42] By using this method on the CP surface, BAC can be adsorbed from single proteins (antigens or antibodies) until whole cells.[30]

Covalent attachment is the next approach that has been used for immobilization of BAC on the surface of conducting polymer.[42] Covalent attachment of BAC on the surface of CP resulted in higher BAC activity than that obtained previously by adsorption and is responsible for enhanced stability of analytical system during continous measurements. The next very important advantage of this method is ordered BAC orientation, which is especially important for effectivity of immunosensors.

Entrapment. In many cases, irreversible adsorption or covalent binding of an antigen or antibody does not lead to an effective immunosensing system due to the instability of Ag or Ab molecules, low Ab loading, or the potential loss of antigenic activity during covalent immobilization. A more favorable approach is the incorporation of Ab or Ag molecules in an electrically conducting polymer layer. Polypyrrole is suitable for this purpose because it can be prepared easily on miniaturized components; besides, it has a high conductivity and is relatively stable. Many PPy-based systems have been described using enzymes,[14] antibodies, catalytically active dopants, ions, or small organics. Usually entrapment of BAC within the polymeric chains of conducting polymer is carried out during electrochemical polymerization of monomer in the presence of conducting polymer. This method is very attractive because the formation of immunosensor can be done during ‘one step’ procedure and is promising for the formation of multiarray biosensors and affinity sensors.[28] On the other hand, polypyrrole is biocompatible and, hence, causes minimal and reversible disturbance to the working environment.

Molecular imprinting. Within PPy, entrapped BAC molecules can be removed by solvent extraction, and the molecularly imprinted polymer is ready for use.[20]

D. Detection Methods and Techniques Used in Electrochemical Immunosensors

Potentiometric, capacitive, and amperometric transducers have been used for electrochemical immunoassays that indicate the Ag-Ab binding directly.

The mechanism of charge generation and propagation during Ag-Ab binding at CP/BAC electrodes can occur according to:

$$CP^{+}A^{-} + e^{-} \longrightarrow CP^{O} + A^{-} \quad (1)$$

$$CP^{+}A^{-} + K^{+} \longrightarrow CP^{+} + A^{-}K^{+} \quad (2)$$

where: CP^{+} is oxidized, CP^{O}-reduced forms of corresponding conducting polymer (polypyrrole). A^{-} is counteranion; K^{+} is countercation.

According to this mechanism, the current obtained at the BAC-immobilized conducting polymer electrodes occurs *via* the following steps: diffusion of ions to the electrode; charge transfer at the porous CP membrane interface; migration through the polymer membrane; adsorption-desorption of the Ag at the CP solution interface. The slow rate of adsorption-desorption process in the last step is considered to be the rate-determining step. This step can be controlled through the appropriate choice of electrical potential.[5]

Amperometric techniques have been used to monitor Ag-Ab binding in real time without using a labeled compound.[26]

Pulsed-accelerated immunoassay techniques are such techniques where a sensor can be used for the analyte detection in static or flow injection mode by applying pulsed potentials between the sensor surface (or working electrode) and the reference electrode. A polypyrrole-based antibody electrode can be used in combination with pulsed amperometric detection. The current obtained can be directly related to the concentration of the analyte in solution.[44]

Conductometric immunoassays. Conductivity simply provides a measure of the ionic concentration and mobility in a solution. However, conductivity measurements are difficult due to the variable ionic background of clinical samples and the relatively small conductivity changes that are observed in such high ionic strength solutions. A second comparative 'blank' electrode must be used, but variable drift at two separate electrodes possess a universal drawback.[45]

Potentiometric detection. The altered surface potential, at near-zero current flow, generated at an ion-selective electrode provides a route to detect the ionic product of an enzyme reaction. Typically, potentiometry is less sensitive than amperometry, the detection limit usually being of the order of micromoles. Potentiometric electrodes, like many other sensing devices, are influenced by solution conditions such as pH and ionic strength, and in particular suffer from the interference of other ions. In addition, there are fewer enzymatic reactions that can be followed by potentiometric electrode.[46]

Capacitive transducers have been used for the real-time and label-free measurement of the Ag-Ab reaction. They are based on the principle that for electrolytic capacitors the capacitance depends on the thickness and dielectric behavior of a conducting polymer/BAC layer before and after interaction with analyte.[47]

Ion-selective electrodes. Ion-selective electrodes are a well-established method for the measurement of K^{+}, Na^{+}, Ca^{2+}, and pH.[48] There are special practical issues concerning clinical potentiometric devices: electrical shielding is critical and the absence of a zero potential as a set baseline makes two point calibration obligatory. Owning to the log-linear relationship between output and concentration, better precision is, in principle, achievable at the lower concentrations, compared with a linear system.

Field effect transistors (FET). The FET, used in electronics for detecting voltage variation with minimal current drain, is the microfabricated forerunner of the ion-selective field effect transistor (ISFET).[49] Polypyrrole can be applied for immobilization of BAC over the gate of ISFET.[50] Molecularly imprinted polymer membranes in combination with FET can be used.[51] These sensors

can be attractive due to both their robustness and small size.

According to application of labels, electrochemical immunoassays are classified into direct and indirect.

The *direct electrochemical immunoassays* do not require any additional labels. In this method changes in charge densities or conductivities are used for transduction and do not need any auxiliary reaction. It seems that the mechanism of the Ag/Ab interaction at CP-based electrodes involves variation in the capacitive properties of the polymer. To work label free is very attractive, especially for the development of *in vivo* immunosensors because it allows real-time measurement without any additional hazardous reagents if weakly affine antibodies are applied.[8]

In *indirect electrochemical immunoassays* the binding reaction is visualized indirectly *via* an auxiliary reaction by a labeling compound. Amperometric transducers in indirect electrochemical immunoassay are used much more frequently than others. Redox-active compounds can be applied as a label for indication of Ag-Ab binding event because antibodies and antigens are usually not electrochemically active within the desired potential range.[8] For an amperometric immunoassay the labeling redox compound should have the following properties: it should be reversibly electroactive; it should not cause electrode fouling; chemical groups for coupling should be available. Indirect electrochemical immunoassay can be divided into two major types: *non-amplified* and *amplified*.

In non-amplified redox-labeled electrochemical immunoassays the indication of one antigen or antibody molecule will generate one signal equivalent. Because the sensitivity of an amperometric sensor for the redox compound is in the lower micromolar range, this kind of assay makes sense only if the concentration of the analyte to be determined is also in this range.[39] For more sensitive immunoassays amplification principles are necessary. One way to amplify the amperometrical signal is preconcentration step. During this step concentration of redox active compound is increasing many times and only after some time (1 to 5 min.) the measurement starts. Higher amplifications are achieved when redox recycling is applied. Redox recycling can be done in different ways by using electrode-electrode, electrode-enzyme, or enzyme-enzyme couples.[8] Thereby, the redox compound is oxidized and reduced in a cyclic manner so that the indication of one labeled antigen or antibody molecule will generate multiple signal equivalents.

Immunosensors for continous measurements. The major problem of use with the immunosensor for continuous measurements is the stability of Ag-Ab or BAC-analyte complexes. To overcome this problem for dissociation of Ag-Ab complex buffers with extreme pH values, extreme salt concentrations are usually used. Flow injection mode applied together with pulsed-accelerated immunoassay techniques can be applied successfully for continuous measurements.[52]

CONCLUSIONS AND FUTURE TRENDS

The use of conducting polymers in conjunction with bioaffinity reagents has provided a powerful route that expanded the range applications of electrochemical detection and its future development is expected to continue.

From the analytical point of view, Ppy has a few very attractive characteristics: (1) it is biocompatible and, hence, causes minimal and reversible disturbance to the working environment; (2) it is capable of transducing the energy arising from interaction between immune reagents into electrical signals that are easily monitored; (3) it protects electrodes from fouling and interfering materials such as electroactive anions; (4) it can be modified *in situ* in a controlled fashion. The characteristics mentioned above possess great application possibilities for PPy in immunosensors devoted to the direct detection of analyte.

The interaction between the proteins, mainly negatively charged at neutral pH, and the delocalized positive charges along the polymer chains induces changes capacitance of the material. Consequently, such interactions, evidenced from electrochemical measurement, are the basis of bioaffinity signal. The use of a wide range of counterions will provide significant change in affinity at the conducting polymer ion-exchange sites. The application of microelectrode technologies already established in electronic nose devices will

be beneficial to PPy-based immunosensors. Further exploitation of this technology to immobilize bioaffinity reagents with the polymer matrix may enable the design of smaller, more compact, and portable sensing systems.

The PPy is inherently biocompatible material. The high water content ensures that the surface energy of these materials is such that minimum disturbance is caused for the biologically active compound. In recent years, many assay configurations and transducers have been designed allowing us to detect the analyte at very low concentrations and with high precision. However, for practical applications, a few main problems remain to be solved: (1) for one-way immunotests the production must be so reproducible that calibration-free measurements can be performed; (2) for continuous or quasicontinuous measurements of an analyte the problem of regeneration has to be solved because extreme pH values, extreme salt concentrations, or other factors thaat are usually used for dissociation of complexes BAC with analyte leads to destruction of BAC or polymeric immobilization matrix or distortion of sequence analytical signals. Approaches for overcoming these problems are still in their infancy. It can be predicted that the manufacture of composites consisting of molecularly imprinted polymers and conducting polymers results in obtaining materials that exhibit both predetermined selective molecular recognition and electrical conductivity. This type of material will be of special interest for use in the field of sensor technology.

Future immunosensors will require deliberate control of the molecular structure at the surface of the electrode to meet specific applications. As the surface microstructure becomes more complicated, all chemical methods of construction will be required. These methods use "molecular technology" instead of the bulk technology used at present. In addition, for the construction of more complex microstructures, some degree of molecular self-assembly will be needed and conducting polymers become the new applications. In the future, when common problems for label-free affinity sensors such as limitations for determination of low-molecular-weight analytes and matrix effects have been solved, electrochemical immunosensors based on polypyrrole could have a great potential for direct immunosensing.

The resolution of some of the problems discussed and the production of robust designs, vital for medical, environmental, and other applications, can be based on the application of a CP-like polypyrrole.

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